INVOLVEMENT OF ETHYLENE AND ENDO-β-MANNANASE IN LETTUCE SEED GERMINATION AT HIGH TEMPERATURE

By

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And God said:

"Let the earth bring forth grass, and yield plants bearing seed, and trees bearing fruit". The earth did so and God saw that it was good.

Genesis 1:11

To my wife, Anna Christina, and my daughters, Fabiana, Andressa, and Eduarda

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The lettuce seed endosperm can delay or prevent germination at high temperature by acting as a physical barrier to radicle protrusion. Weakening of the endosperm tissue around the radicle tip is necessary for germination at high temperature. The objective of this research was to investigate the association of ethylene and endo-β-mannanase with lettuce endosperm weakening and germination at high temperature. Several factors that may alter thermotolerance, such as genotype, seed maturation conditions, light, ethylene, priming, and vigor were used to elucidate the involvement of ethylene and endo-β-mannanase in endosperm weakening. Thermotolerant genotypes ('Everglades' and PI 251245) had more ethylene evolution and endo-β-mannanase activity prior to radicle protrusion at high temperature (35°C), than thermosensitive genotypes ('Dark Green Boston', 'Valmaine', and 'Floricos 83'). 'Everglades' and 'Dark Green Boston' pollinated and matured under 30/20°C day/night temperatures had more endo-β-mannanase and

germination at 35°C compared to those matured at 20/10°C. In thermotolerant 'Everglades' germination in the dark lead to reduced ethylene evolution and endo-β-mannanase activity compared to germination in the light (prior to radicle protrusion). The ethylene precursor, 1-aminocyclopropane-1-carboxilic acid, applied during seed germination at 35°C or during priming, lead to increased endo-β-mannanase activity prior to radicle protrusion and circunvented thermoinhibition in 'Dark Green Boston'. Conversely, the ethylene action inhibitor, silver thiosulphate, applied during seed germination at 35°C or during priming lead to decreased ethylene evolution, endo-β-mannanase activity, and seed germination at high temperature in both 'Dark Green Boston' and 'Everglades'. Endo-β-mannanase activity was detected during priming (without radicle protrusion). Priming led to retained endo-β-mannanase activity and circumvented thermoinhibition of 'Dark Green Boston'. Aged seeds of 'Everglades' had lower ethylene evolution and endo-β-mannanase activity prior to radicle protrusion and germinated at 60% at 35°C, whereas nonaged seeds had more ethylene evolution and endo-β-mannanase activity and germinated 100%. A relationship between ethylene production and endo-β-mannanase activity prior to radicle protrusion suggested that they induced weakening of the endosperm allowing germination of lettuce at high temperature.

CHAPTER 1
INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops produced for fresh market in the United States. The production of lettuce in 1997 was 87 millions cwt. on 277,680 harvested acres, representing approximately 1,6 billions US dollars in value (USDA, 1998). Lettuce is grown year around in the United States; however, continuous high temperature conditions during certain seasons when sowing either into the field (direct seeding) or in the greenhouse (transplant production) can lead to reduced or erratic seed germination. This can directly reduce yields and grower profits, especially of once-over harvested crops.

Lettuce seed germination is strongly temperature dependent. The optimum temperature for germination is around 20°C, and most lettuce genotypes fail to germinate at temperatures above 30°C. When high temperature conditions occur during seed imbibition, two different phenomena may occur: 1. thermoinhibition, a reversible condition, since germination will occur when the temperature decreases to a suitable level, and; 2. thermodormancy, where seeds do not germinate after the alleviation of high temperature. In this case, however, germination can occur if seeds are treated with growth regulators or subjected to priming.

Different strategies to alleviate the problems of thermoinhibition and/or thermodormancy have been used. Some thermotolerant genotypes have been developed, but environmental effects on expression of tolerance have been observed. Environmental

factors during seed maturation can also influence the threshold temperature for seed germination. Seed priming has been successfully used to overcome the problem of high temperature inhibition of lettuce seed germination. Under laboratory conditions, exogenous hormones have significantly improved lettuce seed germination at high temperatures. Nonetheless, the physiological and biochemical processes that control lettuce seed dormancy at high temperature are still not largely understood.

The lettuce seed embryo is completely enclosed within two to- four-cell layer endosperm comprised mainly of galactomannan polysaccharides. The endosperm may delay or prevent germination by acting as a physical barrier to radicle protrusion, especially under unfavorable conditions. Thus, weakening of the endosperm layer of lettuce seeds is a pre-requisite to radicle protrusion at high temperatures. Since the lettuce endosperm cell walls are composed largely of mannans, endo-β-mannanase could be a potential regulatory enzyme in endosperm weakening.

The objective of this research was to investigate the involvement of ethylene and the enzyme endo-β-mannanase in lettuce seed germination under high temperature conditions. In an attempt to elucidate the relationship between these factors, several approaches were taken. These were: 1) studying the interaction between light and temperature on seed germination; 2) testing genotypes that have exhibited different capacities to germinate at high temperature; 3) verifying the seed priming effect; 4) using inhibitors of ethylene synthesis and action during seed germination; and 5) comparing different seed lot quality.

CHAPTER 2
REVIEW OF LITERATURE

In this review, the effects of temperature on lettuce seed germination are addressed. The importance of lettuce endosperm to seed germination, especially at high temperature, is also described. Evidence for a role of endo-β-mannanase and other enzymes associated with endosperm weakening and seed germination are discussed. Finally, ethylene involvement during seed germination is addressed. High temperatures during seed imbibition might inhibit ethylene production and endo-β-mannanase activity needed for lettuce seed germination. The purpose of the following review was to examine the literature for information addressing the involvement of the ethylene and the enzyme endo-β-mannanase in lettuce endosperm weakening and, consequently, seed germination under high temperature conditions.

Temperature Control of Lettuce Seed Germination

Since early studies of the effects of high temperatures on lettuce seed germination were reported (Borthwick and Robbins, 1928), the aspects of thermoinhibition and thermodormancy have been a subject of interest to many researchers. Some lettuce seeds can germinate at temperatures ranging from 5 to 33°C (Gray, 1975). Different cultivars vary in their response to germinate at high temperature (Harrington and Thompson, 1952, Thompson et al., 1979). The optimum temperature for germination of most lettuce genotypes is around 20°C (AOSA, 1993). In general, temperatures above 30°C adversely

affect lettuce seed germination. High temperatures may decrease either germination rate or germination percentage. During imbibition, temperatures above the optimum for a specific cultivar may lead to two different phenomena: thermoinhibition, a reversible process, where the seeds do not germinate above a certain temperature, but will germinate after moving to a lower temperature. Prolonged exposure to supraoptimal temperatures may induce a true dormancy, a phenomenon known as thermodormancy or secondary dormancy (Khan, 1980/81). In this case, the seeds will not germinate even when the temperature is reduced to a favorable range. Thus, dormancy must be overcome for the seeds to germinate. Lettuce seeds are particularly sensitive to high temperature during the first hours of imbibition (Gray, 1977). For example, seeds imbibed at low temperature for a certain period of time might germinate when later placed at high temperature (Takeba and Matsubara, 1976), suggesting that thermoinhibition and thermodormancy can be bypassed prior to radicle protrusion.

Light is another factor that can affect lettuce seed germination (Borthwick et al., 1952; Bewley and Black, 1994). For example, seed dormancy in lettuce may be induced when seeds are imbibed for a prolonged period in the dark (Toole et al., 1956). This effect may be alleviated if red light is applied to the imbibing seed early in the germination process (Scheibe and Lang, 1965). Several researches have shown that lettuce seeds imbibed at high temperature are dependent on red light for germination (Evenari et al., 1953; Vidaver and Hsiao, 1974; Heydecker and Joshua, 1977; Georghiou and Thanos, 1983; Saini et al., 1989). Fielding et al. (1992) reported that there was a close link between phytochrome action and the upper temperature limit for lettuce seed germination.

Different factors have been attributed to explain the failure of lettuce seeds to germinate at high temperature. These include the impermeability of seed coverings to gases (Borthwick and Robbins, 1928) and water (Speer, 1974), nonfunction of phytochrome (Scheibe and Lang, 1969), inhibitory effects of abscisic acid (McWha, 1976), deficiency of the growth potential of the embryo (Nabors and Lang, 1971a), inhibition of the secretion of cell-wall degrading enzymes (Ikuma and Thimann, 1963), and mechanical restraints of the seed coverings (Ikuma and Thimann, 1963). More recently, considerable evidence suggests that the lettuce endosperm layer can directly restrict radicle protrusion, especially at high temperature (Sung, 1996; Sung et al., 1998a). However, the exact mechanism of thermoinhibition or thermodormancy continues to be debated.

The Lettuce Endosperm

The lettuce seed (achene) embryo is completely enclosed within the endosperm that the growing radicle must penetrate for the seed to germinate. The lettuce endosperm, unlike that of several other species, is a living tissue, and comprises about 8% of the seed dry weight. Most of the lettuce endosperm consists of a distinct double layer of cells, except at the micropylar region where there are three or more cell layers (Borthwick and Robbins, 1928). The endosperm cell walls are comparatively thick. The part of the cell wall next to the integument is consistently thicker (25-30 μm) than the inner walls (6 –10 μm) of the endosperm (Jones, 1974). Unlike most plant cell walls, the contours of the wall of the lettuce endosperm are highly irregular, and numerous wall protuberances project into the cytoplasm (Jones, 1974). The dense cytoplasm of the endosperm contains

organelles of protein and lipid storage (0.23 and 4.3 % of seed dry weight, respectively) (Jones, 1974; Halmer et al., 1978; Leung et al., 1979).

The lettuce endosperm cell wall comprises two-thirds of the total seed cell wall polysaccharide material (Halmer et al., 1975). It is composed largely of mannose-containing polysaccharides (58 – 74%) (Halmer et al., 1975, Dutta et al., 1994), probably (1,4)-β-mannans (Bewley et al., 1983). Some galactose (approximately 10%) is also present, suggesting the presence of galactomannans (Bewley et al. 1983). This hemicellulose consists of a linear backbone of β-1,4-linked mannose units with branches of single β-1,6-linked galactose residues (Ouelette and Bewley, 1986). Other sugars, such as rhamnose, fucose, arabinose, xylose, glucose and uronic acids are also present in lettuce seed endosperm cell walls (Halmer et al., 1975; Dutta et al., 1994). The micropylar cell walls of lettuce endosperm are compositionally different from the lateral region. Cell walls from the micropylar region have a significantly higher proportion of arabinose (28%) and glucose (15%) (although mannose is still the predominant sugar) compared to walls prepared from the lateral region, which consisted mostly of mannans (75%) (Dutta et al., 1994).

Lettuce endosperm is the initial source of food reserves for the growing embryo. Mobilization of the endosperm requires a number of enzymes that may be stored or synthesized de novo within the endosperm cells. The cells of lettuce endosperm contain all of the cytological apparatus for enzyme synthesis (Jones, 1974). The galactomannan-rich polysaccharides are important food reserves utilized by the growing embryo after germination, but before the mobilization of the major reserves stored in the cotyledons (Halmer et al., 1978; Leung et al., 1979). Most of the carbohydrate for the growing

lettuce embryo comes from the degradation of cell wall polysaccharides (Park and Chen, 1974) that are subsequently converted to sucrose and transported to the embryo, primarily into the cotyledons (Park and Chen, 1974; Halmer et al., 1978).

Galactomannans are also found as a major component of the endosperm tissue of a number of species from *Leguminosae* (Ganter et al., 1995; McCleary and Matheson, 1975; Reid and Meyer, 1970; Leung et al., 1981; Buckeridge and Dietrich, 1996) *Palmae* (Samonte et al., 1989; Daud and Jarvis, 1992), *Anonacea*, *Rubiaceae* (Giorgini and Comoli, 1996), *Araceae* (Nishinari et al., 1992) and *Convolvulaceae* (McCleary and Matheson, 1975). In most of these species, galactomannans are hydrolyzed in the post-germinative period to their monosaccharide constituents (mannose and galactose), which are then used by the growing embryo.

Aside from the endosperm's importance as a food reserve, the structural complexity of the endosperm cell walls in lettuce has been correlated with the role this tissue plays in restricting early embryo growth.

Mechanical Restraint of the Endosperm to Germination

Seeds develop from fertilized ovules and are usually comprised of three genetically different components: the embryo, the endosperm, and the seed coat. Some species contain perisperm as storage tissue instead of endosperm, and some species may contain both tissues. Generally, these tissues enclose the embryo. All of these seed-enclosing structures may act as physical barriers interfering with the growth and emergence of the embryo. In many species, the seed coat, developed from the integument of the ovule, is the main protective barrier between the embryo and the external environment. The role of

the seed coverings in seed germination was investigated in other species, including muskmelon (Welbaum et al., 1995), pepper (Watkins and Cantliffe, 1983), and tomato (Groot and Karssen, 1987).

In lettuce, the control of germination, especially at high temperature, appears to be exerted within the layers surrounding the embryo of which there are three: the pericarp, integument, and endosperm (Borthwick and Robbins, 1928). The outermost coat is the longitudinally ribbed, non-living pericarp, of maternal origin (Drew and Brocklehurst, 1984). Between the endosperm and the pericarp is the integument, a composite but delicate layer with semi-permeable properties. The lettuce endosperm has been thought to provide a mechanical resistance against radicle expansion, since seed germination was increased by puncturing or removing the endosperm (Borthwick and Robbins, 1928; Evenary and Neuman, 1952; Ikuma and Thimam, 1963; Prusinski and Khan, 1990).

A number of different features might count for the resistance to germination imposed by the endosperm. The thick cell walls and dense cytoplasm of endosperm cells contribute to the strength of the endosperm layer (Ikuma and Thimann, 1963; Tao and Khan, 1979). Halmer et al. (1976) reported that these thick cells act as a barrier to seed germination, especially under high imbibition temperatures. The chemical composition of the cell walls may also affect the rigidity of the endosperm. It is known that galactomannans in the endosperm cell walls of the legume seeds result in a hard seed coverings (Zamski, 1995). Also, the cell wall column-like projections which mainly extend from the external cell wall to the internal wall (Borthwick and Robbins, 1928) add rigidity to the tissue (Psaras, 1984). The endosperm, possibly with the attached integumentary membrane, can exert a mechanical resistance to embryo expansion (Pavlista and Haber,

1970). In another study, Drew and Brocklehurst (1984) suggested that the pericarp plays a role in the regulation of germination. Wurr et al. (1987) verified that the force required to penetrate the lettuce pericarp was on average more than twice that required to penetrate the endosperm. In their study, the correlation between seed germination at high temperature and seed penetration force was genotype-dependent. Tao and Khan (1979) reported that changes in the strength of the endosperm were not directly related to the protrusion of the radicle. However, Sung et al. (1998a) using a puncture test, concluded that the lettuce endosperm represented the source of resistance to radicle growth at high temperature.

Endosperm Weakening

Nondormant seeds enter in the third phase of germination (radicle protrusion), resulting in the completion of germination. For germination to be completed, the radicle must expand and penetrate the surrounding tissues. Three possible causes for radicle growth initiation have been suggested: 1) solute accumulation (which lowers the osmotic potential in the radicle cells and increases embryo turgor); 2) cell wall loosening (which increases extensibility) of the radicle, or 3) weakening of the tissues surrounding the radicle tip, thus allowing it to elongate (Bewley and Black, 1994; Bradford, 1995).

In lettuce, two different mechanisms for overcoming the mechanical restraint of endosperm at high temperature have been postulated: 1) the mechanical force of the growing embryo, which exerts pressure against the endosperm envelope, and 2) weakening of the endosperm tissue. It is possible that both mechanisms operate together in allowing lettuce seed to germinate.

Radicle protrusion in lettuce results from cell elongation rather than cell division (Haber and Luippold, 1960; Bewley and Black, 1994). The different sequences of the inception of cell division and cell expansion are dependent on temperature, indicating different mechanisms for germination at different temperatures (Haber and Luippold, 1960). For example, at high temperature, mitosis may occur before cell expansion. A negative water potential in the cells of the embryo is essential for seed germination and is the principal force for cell expansion. The ability of the embryo to absorb water and to initiate growth is dependent on the osmotic potential of its cells (Nabors and Lang, 1971a, 1971b; Takeba, 1980). The differences in water potential in the embryonic axis from the accumulation of osmotically active compounds (Carpita et al., 1979; Takeba, 1980) was considered sufficient for radicle protrusion and embryonic axis elongation (Nabors and Lang, 1971a, 1971b; Bewley and Halmer, 1980/81). Thus, increasing the growth potential in the embryo might be a way to overcome the mechanical resistance of endosperm. Takeba and Matsubara (1979) suggested that at high temperature, lettuce seeds were unable to germinate because the embryonic growth potential was not sufficient to overcome the restraining force of the seed coat. However, thermoinhibition of lettuce seed germination is not due to a failure of the embryo to absorb water or develop sufficient turgor (Bradford, 1990; Weges et al., 1991). In a study using chlorine treatments during lettuce seed imbibition, Pavlista and Haber (1970) observed considerable embryo elongation within the confines of the endosperm without protrusion of radicle. Thus, other mechanisms might be involved in radicle protrusion at high temperature.

Structural and morphological changes occurring in the endosperm prior lettuce seed germination (Georghiou et al., 1983, Psaras et al., 1981) suggest another hypothesis

for radicle protrusion. Georghiou et al. (1983) observed structural changes opposite the radicle end in lettuce seeds germinating under red light. Modification of the cytoplasm of endosperm cells in the micropylar region was observed before radicle protrusion, and these changes were a prerequisite for the completion of lettuce seed germination (Georghiou et al., 1983). The absence of structural changes in the endosperm cells at high temperature could provide the basis for the induction of secondary dormancy (Georghiou et al., 1983). Takeba and Matsubara (1977) found that small fat bodies from lettuce seed cells disappeared from lettuce seeds during early stages of imbibition at 20°C, but not at 35°C. Phytochrome destruction and/or reversion to an inactive form (Toole, 1973), or the inactivation of a thermolabile factor (Takeba and Matsubara, 1976), could explain the absence of structural changes in endosperm cells at high temperatures. In addition, Pavlista and Valdovinos (1978) observed disruptions in lettuce endosperm before germination occurred. In an extensive study, Sung (1996) verified that endosperm cells in the micropylar region were structurally altered during germination at high temperature. Changes included separation of the endosperm layer from the integument, depletion of protein bodies, formation of empty vacuoles, cytoplasm condensation, and rupture of the endosperm cell walls with subsequent embryo growth toward this opening. Sung (1996) concluded that weakening of the endosperm layer was a required prerequisite to radicle protrusion.

Weakening of the endosperm has also been reported in other species, (Watkins and Cantliffe, 1983; Karssen et al., 1989; Dahal and Bradford, 1990; Groot and Karssen, 1992). In the last few years evidence has emerged showing that some seeds germinate via enzymic degradation of the endosperm. Weakening of the endosperm in the micropylar

region prior to radicle protrusion was also observed in anatomical studies of pepper (Watkins et al., 1985) and *Datura ferox* (Sanchez et al., 1990). Evidence that weakening of the endosperm could be a prerequisite to radicle protrusion was provided for tomato seeds (Groot et al., 1988; Ni and Bradford, 1993; Leviatov et al., 1995; Nomaguchi et al., 1995). In these species, endosperm weakening was suggested to be mediated by endo-β-mannanase. The increase in endo-β-mannanase activity was linearly correlated with decreasing resistance of endosperm to penetration (Hilhorst and Karssen, 1992). However, endo-β-mannanase activity in the endosperm cap of tomato seeds was not sufficient to permit seeds to complete germination (Toorop et al., 1996). Production of hydrolyases within the endosperm and secretion into the cell walls, causing weakening and allowing the radicle to protrude has been proposed for other species (Bewley, 1997a; Black, 1996).

In lettuce, Ikuma and Thimann (1963) proposed that the action of an enzyme produced by the embryo enables the radicle tip to penetrate through the restricting tissues. Possible chemical weakening of the endosperm near the radicle end was also suggested (Pavlista and Haber, 1970). Other enzymes, including cellulase, pectinase, and pentosanase, were effective at promoting germination of dormant seeds of lettuce (Ikuma and Thimann, 1963). Increased activity of carboxymethylesterase prior to endosperm degradation has also been reported (Pavlista and Valdovinos, 1975). However, Bewley (1997b), has claimed that lettuce seeds do not produce cellulase.

Since lettuce endosperm cell walls are composed largely of galactomannans (Halmer et al., 1975), endo-β-mannanase might be the enzyme most likely involved in the cell wall degradation leading to endosperm weakening and subsequent radicle protrusion.

Endo-β-Mannanase Activity

The time of appearance of endo-β-mannanase activity during germination has been largely debated. In some studies, mannanase activity detected in dry seeds or after the first hours of imbibition might have been due to activation of preexisting enzyme (Dutta et al., 1994) or the retention of enzyme produced during seed development (Hilhorst and Downie (1995). Thus, some researches have speculated that growth conditions during seed development might affect endo-β-mannanase levels. Imbibed seeds of fenugreek and carob did not exhibit endo-β-mannanase activity until after completion of germination (Reid et al., 1977; Spyropoulos and Reid, 1988; Kontos and Spyropoulos, 1995). In lettuce, early studies detected mannan hydrolysis only as a post-germinative event (e.g., after radicle protrusion), (Bewley and Halmer, 1980/81; Halmer et al., 1975, 1976). For example, mannanase activity increased about 100 fold in all regions of the endosperm during 15 hours following germination (Halmer et al., 1978). Recently, Dutta et al. (1997) reported that a cell-wall-bound endo-β-mannanase is expressed in lettuce seed endosperm prior to radicle protrusion and is regulated by the same conditions that govern seed germination. These authors suggested that this enzyme is likely to be involved in the weakening of the endosperm cell walls.

Lettuce endosperm cell walls exhibit endo-hydrolase activity (Dutta et al.,1994). These endo-hydrolases are highly substrate specific (Huber and Nevins, 1977). Endo-β-mannanase is targeted toward the mannan-rich endosperm cell walls (Halmer et al., 1975, 1976). Experiments have shown that endo-β-mannanase (EC 3.2.1.78) is synthesized *de novo*, beginning after 6 hours from the start of lettuce seed imbibition, and continues to do

so far for approximately 12 hours more (Halmer, 1989; Bewley and Halmer, 1980/81). Estimates of molecular weight by chromatography and electrophoresis were 46,000. Endo-β-mannanase is a major protein secreted by lettuce endosperm (Halmer, 1989). Two-dimensional PAGE indicated that mannanase exists as three isoforms, with pIs between 4.75 and 4.9. Maximum enzyme activity was achieved at pH 5.0 (Downie et al., 1994; Dutta et al., 1997). However, low enzyme activity represented by the cell wall has been reported in lettuce (Dutta et al., 1994, 1997). This low activity may be due to limited number and specific nature of the bonds susceptible to catalysis during autolysis within lettuce endosperm (Dutta et al., 1997). Also, endo-β-mannanase is tightly bound to the endosperm cell walls. More structural details of matrix polysaccharides and their linkages between the polymers of endosperm cell walls need to be known.

Lettuce genotypes differ in endo-β-mannanase abundance and isozyme complements (Dirk et al, 1995). No isozymes could be detected when the lettuce embryo and endosperm were separated after 4 hours of imbibition (Dirk et al, 1995). Similar results were found in tomato seeds, where fewer isoforms were produced when embryo and endosperm were dissected prior to completion of germination and incubated separately (Voight and Bewley, 1996).

The differences in the pI values of the isozymes in the seeds and plant parts of several crops suggests that endo-β-mannanase is variable in its amino acid composition, or in the glycosylation patterns, or both (Dirk et al, 1995). A cDNA encoding a (1,4)-β-mannanase from endosperm of germinated tomato seeds has been recently isolated and characterized (Bewley et al., 1997). Also in tomato, multiple isozymes of endo-β-mannanase were reported in germinating seeds (Dirk et al., 1995; Nonogaki et al., 1995;

Toorop et al., 1996; Voigt and Bewley, 1996). In this species, the enzyme isoforms produced in the micropylar and lateral regions were different (Nonogaki and Morohashi, 1996; Voigt and Bewley, 1996). It is possible that only specific isozyme(s) are regulated in a quantitative manner with germination rates (Dahal et al., 1997). Multiple forms of endo-β-mannanase were also isolated from the endosperm of different legume seeds (McCleary and Matheson, 1975). For instance, several isozymes of mannanase were detected in the endosperms of fenugreek and carob (Kontos et al., 1996).

Cell walls isolated from lettuce endosperm are capable of autohydrolytic activity, indicating the presence of cell-wall-bound hydrolases (Dutta et al., 1994). Endosperm is the site of mannanase production, although it is controlled by the embryo (Halmer and Bewley, 1979). In fenugreek, Spyropoulos and Reid (1985) and Malek and Bewley (1991) also reported the involvement of the embryonic axis in the regulation of endosperm mobilization. Mannanase production is subject to inhibition. The endosperm may contain some inhibitor(s) that needs to be removed in order to have enzyme synthesis. Some studies have demonstrated that the volume of buffer used during incubation of isolated endosperms affects the production of endo-β-mannanase. For example, isolated endosperms incubated in 1mL produced substantial quantities of mannanase, whereas those incubated in 20μL did not (Dulson et al., 1988; Dulson and Bewley, 1989). Dulson et al. (1988) concluded that endogenous abscisic acid (ABA) regulated mannanase production in isolated lettuce endosperms. In another study, treatment with ABA suppressed both lettuce germination and endo-β-mannanase activity (Dutta et al., 1997). Abscisic acid has also been reported to inhibit both germination and endo-β-mannanase activity in tomato (Hilhorst and Downie, 1995; Nomaguchi et al., 1995; Nonogaki and

Morohashi, 1996; Voigt and Bewley, 1996) and fenugreek (Malek and Bewley, 1991) seeds. Endo-β-mannanase activity inhibition by ABA in tomato seeds was dependent on tissue localization. Abscisic acid was incapable of inhibiting endo-β-mannanase activity in the endosperm cap, but did inhibit activity in the lateral endosperm (Toorop et al., 1996). However, Dahal et al. (1997) reported that ABA did not act by inhibiting the induction of mannanase activity in tomato seeds and concluded that ABA can prevent endosperm weakening in the absence of the embryo.

Endo-β-mannanase activity is also regulated by environmental factors. Red light-treated seeds of lettuce produced high amounts of endo-β-mannanase but only after radicle protrusion (Bewley and Halmer, 1980/81). Conversely, seeds imbibed in the dark produced little mannanase and did not germinate (Halmer and Bewley, 1979). Endo-β-mannanase was strongly promoted in the micropylar region of *Datura* seed endosperm under red light (Sanchez and Miguel, 1997). Imbibition of lettuce (Dutta et al., 1997) and tomato (Leviatov et al., 1995) seeds, respectively, at high or at low temperature, decreased enzyme activity. Incubation of lettuce cv. Pacific seeds at high temperatures resulted in total prevention of germination and almost complete suppression of endo-β-mannanase (Dutta et al., 1997). Studying different tomato genotypes, Leviatov et al. (1995) verified that the expression of increasing endo-β-mannanase activity in the micropylar region of the endosperm at low temperature was characteristic of cold tolerant germinating lines. In this study, there was a positive relationship between germination ability at low temperatures and endo-β-mannanase activity in the six progeny lines. In a different study, Downie et al. (1997) reported increased endo-β-mannanase activity in white spruce seeds during dormancy alleviation by chilling.

Gibberellins also affect endo-β-mannanase synthesis. Ikuma and Thimann (1963) suggested that germination of lettuce seeds promoted by gibberellin was due to its stimulation or activation of hydrolytic enzymes. Treatment with gibberellin was accompanied by significant enhancement of endo-β-mannanase activity in lettuce seeds (Halmer and Bewley, 1979), and *Datura* (Sanchez and Miguel, 1997). In the later study, hydrolase activity and germination were blocked by paclobutrazol, an inhibitor of gibberellin synthesis. Using gibberellin-deficient *gib-1* mutant tomato, Groot et al. (1988) concluded that the weakening of endosperm prior to radicle protrusion was mediated by a gibberellin-induced enzymatic degradation of the mannan-rich cell walls. In lettuce, alleviation of thermoinhibition with gibberellin was accompanied by significant enhancement of mannanase activity (Dutta et al., 1997).

It has been well documented that seed priming, an osmotic treatment, circumvents thermodormancy of lettuce seeds and allows germination at higher temperatures (Guedes and Cantliffe, 1980; Cantliffe et al., 1981; Khan et al., 1980/81; Wurr and Fellows, 1984; Valdes et al., 1985). Increased activity of endo-β-mannanase (Karssen et al., 1989) and galactomannan hydrolyzing enzyme (Nonogaki et al., 1992) was observed during seed priming of tomato. The endosperm layer of lettuce seeds was weakened by priming (Sung, 1996).

Whether endo-β-mannanase, in itself is responsible for germination, or works in combination with other factors has been an open question. In tomato, endo-β-mannanase can increase in the absence of radicle protrusion, or vice-versa (Bewley, 1997a). In addition, the amount of endo-β-mannanase necessary to ensure endosperm weakening has not been determined. Thus, although endosperm weakening is likely to be essential for

seeds to complete germination, how this is achieved remains unknown. In lettuce seeds, Dutta et al. (1994) verified during autolysis, a substantial release of arabinose and galactose, in addition to mannose. Thus, endo-β-mannanase may not be rate limiting and additional wall-hydrolyzing enzymes other than endo-β-mannanase may be involved in the lettuce endosperm weakening.

Other Enzymes Associated with Endosperm Weakening and Seed Germination

Galactomannans consist of a backbone of β-1,4-linked mannose subunits with β-1,6-galactose side chains. This polymer requires three different enzymes for its complete mobilization: endo-β-mannanase, α-galactosidase (EC 3.2.1.22), and -β-mannanosidase (β-mannosidase or exo-β-mannanase, EC 3.2.1.25) (Ouellette and Bewley, 1986; Bewley et al., 1983). These enzymes may originate in different regions of the seed and they all behave differently during germination. The β-1,4-linked mannan backbone is hydrolysed via the action of endo-β-mannanase; and the β-1,6-galactose side chains are released by α-galactosidase. β-mannosidase further catalyses the hydrolysis of the oligomannans produced by endo-β-mannanase. α-galactosidase, an exo-polysaccharase, and endo-β-mannanase act cooperatively to effect the hydrolysis of the lettuce endosperm cell walls (Leung and Bewley, 1983). β-mannosidase occurs during lettuce seed germination and is present exclusively within the cotyledons (Bewley et al., 1983). The likely substrates for this enzyme are the products of endosperm cell wall mobilization, mannobiose and mannotriose, which diffuse to the cotyledons (Ouellette and Bewley, 1986; Bewley and Halmer, 1980/81). The synthesis of β-mannosidase could be induced by its substrate, or by some factor(s) released from the degrading endosperm (Bewley and Halmer, 1980/81).

Glycosidases are not believed to be responsible for in situ weakening of wall structure (Dutta et al., 1997). The galactomannan polysaccharides from different species have different proportions of D-galactose and D-mannose, but always consist of a β (1-4) mannan backbone with single D-galactose branches linked β (1-6), (Smith and Montgomery, 1959). The D-galactose content can vary from 10 to 50% according to the species (McCleary and Matheson, 1975).

β-mannanase has limited ability to hydrolyze galactomannans with high galactose contents (McCleary and Matheson, 1975). Seeds containing these galactomannans had very active α-galactosidases. In dry carob seeds there is substantial activity of α-galactosidase (Kontos and Spyropoulos, 1995), while in *Senna occidentalis* (Edwards et al., 1992), α-galactosidase is present during seed development. Thus, it has been suggested that in developing seeds the action of this enzyme reduces the high galactose/mannose ratio of the newly synthesized galactomannan (Kontos and Spyropoulos, 1996). Other cell wall enzymes with putative functions in seed germination have also been reported. In muskmelon, besides endo-β-mannanase, endo-1, 4-β-glucanase activity was observed about 5 hours before radicle protrusion (Welbaum and Wang, 1997). β-1,3-glucanase was reported in tobacco seeds, increasing in the micropylar region of the endosperm before radicle protrusion (Leubner-Metzger et al., 1996). Expression of a putative arabinosidase mRNA has also been detected in tomato seeds (Dahal et al., 1997). All these studies show evidence for enzymatic mechanisms for weakening the tissues constraining the embryo, allowing the radicle to protrude from the seed. Making inferences from those studies, it is reasonable to experimentally match the

cell wall composition of the restraining tissue, in this case, lettuce endosperm, with the probable major enzyme involved, in this case, endo-β-mannanase.

Ethylene and Lettuce Seed Germination

Ethylene can stimulate germination and overcome dormancy in many seeds (Abeles et al., 1992; Esashi, 1991). The inhibitory effect of high temperature on lettuce seed germination for example, is overcome by exogenous ethylene (Abeles and Lonski, 1969; Burdett, 1972a; Negm et al., 1972; Keys et al., 1975; Rao et al., 1975; Dunlap and Morgan, 1977; Fu and Yang, 1983; Abeles, 1986; Saini et al., 1986; Khan and Prusinski, 1989; Saini et al., 1989; Huang and Khan, 1992). Despite the accepted involvement of ethylene in seed germination, the mechanistic details are poorly understood.

High temperatures (35 to 40°C) inhibit ethylene production in a number of plant tissues (Yu et al., 1980). For instance, the negative effect of high temperature on chickpea seed germination was due to low ethylene production (Gallardo et al., 1991). In lettuce, ethylene synthesis or sensitivity to ethylene was decreased at high temperature during seed imbibition (Burdett, 1972a; Dunlap and Morgan, 1977; Abeles, 1986; Khan and Huang, 1988). High temperature appears to inhibit the conversion of 1-aminocyclopropane-1-carboxilic acid (ACC) to ethylene (Khan and Prusinski, 1989). Gallardo et al. (1991) observed that high temperatures decreased the levels of free ACC in chickpea seeds. The conversion of ACC to ethylene decreased as the imbibition temperature of lettuce seed was increased from 25 to 35°C (Prusinski and Khan, 1990). When lettuce seeds were imbibed directly at 35°C, ACC synthesis was not detectable (Huang and Khan, 1992). In apple fruit, an increase in temperature to 35°C caused an accumulation of endogenous

ACC and ethylene production was greatly reduced, suggesting that the conversion of ACC to ethylene was inhibited. The conversion of ACC to ethylene was more sensitive to high temperature inactivation than was ACC synthesis. Thus, the conversion of ACC to ethylene is the primary site of high temperature inactivation.

Although high temperatures may inhibit ethylene production and raise the threshold concentration of ethylene needed for lettuce seed germination, is the thermoinhibition caused by a reduction in the ability of seeds to produce ethylene? Early work by Abeles and Lonski (1969) showed that ethylene did not overcome thermodormancy in lettuce seeds leading to the suggestion that ethylene action was limited to early steps in germination. Also, the reduction in lettuce germination with increasing imbibition temperature was not due to a decrease in pregermination ethylene synthetic capacity of the seeds, since an increase in the rate of ethylene production was not a prerequisite of germination at supraoptimal temperatures (Burdett, 1972a). Therefore, the mechanism of thermoinhibition in lettuce does not appear to be the result of reduced ethylene synthesis (Small et al., 1993). Ethylene had some effect on softening of the endosperm tissue; however this effect was not correlated with the effect of ethylene as a germination promoter (Abeles, 1986). Abeles (1986) suggested that the action of ethylene in lettuce seed germination was the promotion of cell expansion in the embryonic hypocotyl. Dutta and Bradford (1994) also suggested that ethylene acts primarily on the embryo rather than on tissues enveloping it. They reported that ACC (via conversion to ethylene) extended the high temperature limit for lettuce seed germination by acting in the embryo to maintain a water potential sufficiently low to promote the initiation of growth at higher temperatures.

It is evident that ethylene influences biochemical processes in seeds. Ketring (1977) suggested some possibilities for the mechanism of ethylene during seed germination: a) interaction with growth regulators (e.g., ABA) at a basic level of metabolism; b) a combination of growth promoters may be required to maximize a given physiology response; c) a given physiological response is not specific for a single growth promoter; and d) enzyme synthesis and secretion.

Abscisic acid reduces ethylene production by dormant, imbibed peanut seeds and inhibits ethylene production and germination of after-ripened peanut seeds (Ketring and Morgan, 1972). Abscisic acid also inhibited both ethylene production and germination of chickpea seeds (Gallardo et al., 1992) and after-ripened apple embryos (Kepezynski et al., 1977). Exogenous ethylene reversed the inhibitory effects of ABA on dormant seeds (Ketring and Morgan, 1972). The release of dormancy in lettuce seeds by ethylene, however, was not a result of removal of ABA-like compounds (Rao et al., 1975).

Ethylene may interact with light or gibberellin to promote germination at high temperature. For example, ethylene promoted dark germination only in the presence of gibberellin during incubation of lettuce seeds (Dunlap and Morgan, 1977). Gibberellin slightly stimulated ethylene production in peanut seeds (Ketring and Morgan, 1970). The action of gibberellin in lettuce seeds could be through promotion of ethylene synthesis, or ethylene could stimulate germination by a separate mechanism (Stewart and Freebairn, 1969). Burdett and Vidaver (1971) found that both ethylene and gibberellin were necessary to stimulate germination of lettuce seeds at high temperature. However, gibberellin does not promote lettuce germination by stimulating ethylene synthesis (Burdett and Vidaver, 1971). In addition, the reversal of thermodormancy in lettuce by

ethylene occurred only when seeds were incubated in the light (Dunlap and Morgan, 1977).

Heat treatment of *Spergula arvensis* at 30°C prevented ethylene-promoted germination in the dark and the inhibition was reversed by red light (Olatoye and Hall, 1972). The inability of lettuce seeds to germinate at supraoptimal temperatures was due neither to a rapid loss of far red-absorbing phytochrome nor to inadequate ethylene synthesis (Burdett, 1972b). Moreover, Abeles and Lonskin (1969) reported that the ability of ethylene to initiate a small increase in germination of lettuce seeds was apparently not through phytochrome control of ethylene production. It would appear that red light does not promote germination of lettuce seeds by influencing their ability to produce ethylene.

Ethylene evolution from irradiated lettuce seeds began to increase 2 hours prior to radicle protrusion, whereas the dark-incubated (nongerminating) seeds produced a low, constant amount of ethylene (Saini et al, 1989). Thus, endogenous ethylene was essential for the light-induced relief of thermoinhibition of lettuce seed germination. Under osmotic conditions, the promotive effect of ethylene in lettuce seed germination was under the control of phytochrome (Negm and Smith, 1978).

Saini et al. (1986) reported that endogenous ethylene synthesis and action are essential for the alleviation of thermoinhibition of lettuce seeds by combinations of GA_3, kinetin, and CO_2. Negm et al. (1972) showed that CO_2 is required for ethylene action in overcoming thermodormancy in lettuce seeds, but ethylene did not enhance respiration. Cytokinins stimulate ethylene production by some seeds (Khan and Huang, 1988). The relief of salt stress and thermoinhibition of lettuce seed germination by kinetin was accompanied by an enhancement in the pregermination ethylene production. When

cytokinins and ethylene are used together, the stress of high temperature is alleviated in a synergistic fashion (Braun and Khan, 1976; Rao et al., 1975).

Ethylene has also been reported to stimulate the synthesis of some enzymes (Cervantes et al., 1994; Hasegawa et al., 1995). Separation of cells due to the activation of cell wall dismantling enzymes, such as endo- β-1,4-glucanases, was reported recently as an ethylene effect (Casadoro et al., 1998). Other cell wall-degrading enzymes show ethylene-dependency, such as endopolygalacturonase, some isoforms of α-galactosidase, β-arabinosidase, and galactanase (Pech et al., 1998). In some fruits, the climacteric ethylene rise was accompanied by an increase in α-galactosidase and β-mannosidase activities (Moya et al., 1998). Thus, it is reasonably to anticipate that ethylene might overcome the inhibitory effect of high temperature on lettuce seed germination by activating cell wall enzymes responsible for endosperm digestion. The exact timing of ethylene production during seed germination must be determined to clarify this question. Ethylene production by seeds begins immediately after the onset of imbibition and increases with time, however the pattern of ethylene production by seeds during germination differs among species. For example, Takayanagi and Harrington (1971) found only one peak of ethylene production during germination of rape seeds, coinciding with the emergence and elongation of the radicle, cotyledon expansion, and splitting of the seed coat. In oat seeds, ethylene production was observed prior to radicle protrusion and gradually increased (Meheriuck and Spencer, 1964). In lettuce, a major surge in ethylene evolution was observed at the time of visible seed germination (Saini et al., 1986). A peak of ethylene production was also correlated with radicle protrusion (Fu and Yang, 1983). According to Small et al. (1993), however, the major increase in ethylene evolution

occurred after lettuce radicle protrusion. Thus, it appears that this question is still not answered.

The embryo is the major site of ethylene production (Ketring and Morgan, 1969; Esashi and Katoh, 1975). Ethylene concentrations effective at stimulating seed germination of dormant seeds are in the range of 0.1 – 200 µl L-1 depending on the species (Corbineau and Côme, 1995). For lettuce, 10 µl L-1 of ethylene was reported as being close to optimal for promoting seed germination (Burdett and Vidaver, 1971). The differential capacity of different cultivars to produce ethylene during stress generally corresponded with the ability to germinate at high temperature (Prusinski and Khan, 1990). These authors reported that the genotypic variability in seed coat characteristics might influence ethylene production and performance of seeds under stressful conditions. The seed coat may reduce the performance of the growing embryo in two ways under stressful conditions: first, it may serve as a mechanical barrier and, second, it may create a hypoxic environment unfavorable for the conversion of ACC to ethylene (Prusinski and Khan, 1990).

Improved performance of primed lettuce seeds at high temperature is related to high vigor and greater capacity to produce ethylene (Huang and Khan, 1992). Early studies suggested that ethylene may enhance vigor of some seeds and stimulate metabolism of seeds. In peanut and cotton seeds, there was nearly a parallel decrease in vigor and the maximum amount of ethylene produced during germination (Ketring et al., 1974). Takayanagi and Harrington (1971) observed that aged rapeseeds produced less ethylene than do fresh seeds. As rapeseed vigor declined, a delay in attaining maximum ethylene production occurred, and the germination rate of aged rapeseeds was enhanced

by exogenous ethylene treatment. These authors suggested that further aging destroyed the ethylene producing capabilities of the seeds and apparently also destroyed the site of action of ethylene since exogenous ethylene would no longer stimulate growth. In lettuce, seed germination rate was accelerated by ethephon treatment, and practically all of the rate increase occurred during the initial 24 hours after imbibition (Sharples, 1973).

The involvement of ethylene in lettuce seed germination, particularly at high temperature, is still inconclusive. A critical factor is the response of seed germination properties to various ethylene inhibitors. Aminoethoxyvinilglycine (AVG), an inhibitor of ethylene synthesis, has little influence on lettuce seed germination. For example, germination of lettuce seeds at 35°C (Khan and Prusinski, 1989) or at 25°C (Huang and Khan, 1992) was not inhibited by AVG even though it inhibited ethylene production. These results suggest that the seeds that germinated, probably had a very low ethylene requirement, fulfilled by the residual ethylene synthesis occurring in the presence of AVG (Saini et al., 1986). However, AVG or cobalt ions, reduce germination and the effect can be overcome by the addition of ethylene (Abeles, 1986). Also, inhibitors of ethylene action, such as silver and 2,5-norbornadiene, reduce germination and their effect can also be reversed by ethylene. These discrepancies have contributed to the consensus that ethylene plays a vital role in lettuce seed germination.

In summary, lettuce endosperm acts as a physical barrier to radicle protrusion, especially at high temperatures. Under high temperatures, thresholds of weakening of the endosperm may not be attained due to sub-optimal enzyme activity. Endo-β-mannanase should be the most likely enzyme involved in cell wall degradation, leading to weakening of the endosperm and subsequent radicle protrusion. High temperature inhibits ethylene

production, which might be involved in this process. Testing genotypes differing in their level of thermotolerance and/or seed development conditions and providing treatments to enhance and/or inhibit seed germination, could be a good approach to study the involvement of ethylene and endo-β-mannanase during lettuce seed germination at high temperature conditions.

CHAPTER 3
THERMOTOLERANCE IN LETTUCE SEEDS: ASSOCIATION WITH ETHYLENE AND ENDO-β-MANNANASE

Introduction

Under high temperatures, lettuce germination can be erratic or completely inhibited. The exact mechanism of thermoinhibition or thermodormancy is still debated. Some researchers reported that the lettuce endosperm layer mechanically restricts radicle protrusion, especially at high temperature (Halmer et al., 1976; Ikuma and Thimam, 1963). Sung et al. (1998a) concluded that the weakening of the lettuce endosperm layer was a prerequisite to radicle protrusion. Ikuma and Thimann (1963) proposed that the action of an enzyme produced by the embryo enabled the radicle tip to penetrate through the restricting tissues. Since lettuce endosperm cell walls are largely composed of galactomannans (Halmer et al., 1975), endo-β-mannanase might play an important role in weakening of the endosperm and subsequent radicle protrusion. Recently, Dutta et al. (1997) reported that a cell-wall-bound endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion and was regulated by the same conditions that govern seed germination. These authors suggested that endo-β-mannanase was likely to be involved in the weakening of the lettuce endosperm cell walls.

The critical maximum temperature for lettuce seed germination depends on genotype (Harrington and Thompson, 1952; Gray, 1975; Thompson et al., 1979;

Damania, 1986). A wild plant accession, PI 251245, was identified to be a thermotolerant line (Bradford, 1985). However, the thermotolerance character has been obscured by genotype and environment interactions (Nagata, 1997, pers. comm.). Thermotolerant cultivars have been developed (Guzman, 1986; Guzman and Zitter, 1983; Guzman et al., 1992); however, it is not understood how seeds inherit the ability to germinate at high temperature.

During seed development, both genotype and environmental conditions, including temperature, may affect subsequent seed germination at high temperature (Gray et al., 1988; Drew and Brocklehurst, 1990; Steiner and Opoku- Boateng, 1991). Lettuce seeds produced in hot climatic regions germinated better at high temperature (Harrington and Thompson, 1952; Damania, 1986). Under controlled conditions, Gray et al. (1988) verified that lettuce seeds produced at day/night temperature regimes of 30/20°C germinated better at 30°C than seeds produced at 25/15°C or 20/10°C. In another study, Sung et al. (1998a) reported that lettuce seeds matured at 30/20°C had a greater germination percentage at high temperature than those matured at lower temperatures. These authors concluded that the thermotolerance character in lettuce seed was regulated by an interaction between genotype and temperature during seed developement.

Several studies reported that ethylene synthesis was decreased by high temperature during imbibition of lettuce seeds (Burdett, 1972a, 1972b; Dunlap and Morgan, 1977; Abeles, 1986; Khan and Huang, 1988). In addition, exogenous ethylene overcame the inhibitory effect of high temperature on lettuce seed germination (Abeles and Lonski, 1969; Burdett, 1972b; Negm et al., 1972; Keys et al., 1975; Rao et al., 1975; Dunlap and Morgan, 1977; Fu and Yang, 1983; Abeles, 1986; Saini et al., 1986; Khan and Prusinski,

1989; Huang and Khan, 1992). The exact mechanism of ethylene action during lettuce seed germination is not well understood.

The objective of this study was to determine if there was an association among ethylene, endo-β-mannanase, endosperm weakening, and germination of thermotolerant and thermosensitive lettuce genotypes at high temperature.

Materials and Methods

Plant Material

Five lettuce (*Lactuca sativa* L.) genotypes varying in levels of thermotolerance were used in this study: 'Dark Green Boston' (DGB), 'Valmaine' (VAL), and 'Floricos 83' (FLO) (thermosensitive), and 'Everglades' (EVE) and PI 251245 (PI) (thermotolerant). Thermotolerance was defined as the ability of seeds to germinate above 90% at temperatures up to 35°C in light (Guzman et al., 1992; Sung, 1996). All seeds were produced in the same season and region of San Joaquin Valley, California, in 1994. Seeds were stored at 10°C, 40% RH until used.

Seed Maturation Study

Lettuce plants of thermosensitive DGB and thermotolerant EVE were produced under greenhouse conditions until flowering and then transferred to growth chambers at 12 hours photoperiod (day/night) and temperature regimes (day/night) of 20/10 or 30/20°C according to the methods used by Sung et al. (1998a). At maturity, seeds were harvested, threshed, and cleaned manually. Seeds were stored at 10°C and 45% RH until used. DBG and EVE were chosen in this study because the genetic relation between these two genotypes (Guzman et al., 1992).

Seed Germination

Four replications of 25 seeds each were placed on two layers of 5.0 cm diameter germination paper (Anchor Paper, Hudson, WI), moistened with 3 mL of distilled water. Blotters were covered with 5.5 cm petri dish lids and incubated at 20 or 35°C under constant light (fluorescent ~ 26 μmol m^{-2} s^{-1}) on a one-dimensional thermogradient bar (Type DB 5000, Van Dok & De Boer, B.V., Holland). In another study, 'Everglades' seeds were incubated at 20, 27.5, and 35°C under constant light or dark, using the same germination conditions previously described.

Enzyme Activity

A gel-diffusion assay (Downie et al., 1994; Still et al., 1997) was used to measure endo-β-mannanase (EC 3.2.1.78) during seed germination. Gel plates were prepared by dissolving 0.05% (w/v) galactomannan (locust bean gum, Sigma Chemical Co., St. Louis, MO) in incubation buffer (0.1 M citric acid, 0.2 M sodium phosphate, pH 5.0), stirring and heating for 30 minutes. Afterward the solution was clarified by centrifugation at 11,000g for 15 minutes at 4°C. Phytagar (Gibco Lab., Grand Island, NY) at 0.7% (w/v) was added to the clarified solution and stirred and heated to boiling. Thirty mL of the solution were dispensed into 150 x 25 mm disposable petri dishes (Falcon, Franklin Lakes, NJ). After solidification, 32 wells per plate were made using a 2-mm disposable plastic pipette and removing the excised gel by aspiration.

Twenty-eight whole individual endosperms or fourteen pairs (radicle tip + the remaining endosperm, referred to as lateral endosperm) from lettuce seeds imbibed at different temperatures for different periods of time were used on each plate. In one study,

endosperm from seeds imbibed at 20 and 35°C was excised and enzyme activity measured at four different times: after 4 hours of imbibition, 1 hour before radicle protrusion, 1 hour after radicle protrusion, and after 24 hours after imbibition. Radicle protrusion was previously determined in both temperatures for each genotype. Three replications were utilized for each treatment. Endosperms were excised by pressing the cotyledon end using the tip of a conical glass rod. Micropylar and lateral regions were separated with a surgical blade. For seeds incubated in the dark, endosperm excision was performed under a green safelight. Each endosperm, or radicle tip, or lateral part was placed into an individual microtiter plate (Nalge Nunc, Naperville, IL) well containing 20 µL of incubation buffer (0.1 M citric acid, 0.2 M sodium phosphate, pH 5.0) and incubated in the dark for 2 hours at 20°C.

After incubation, 10 µL of buffer from each well was transferred to the gel-diffusion plates and incubated for 24 hours. Petri dishes were covered with a lid and wrapped in Parafilm (American National Can., Greenwich, CT). Gels were stained by adding 10 mL of Congo Red (Sigma Chemical Co., St. Louis, MO) in water (0.4%, w/v) to each plate. Plates were shaken for 20 minutes at 60 rpm during staining. The Congo red solution was decanted and the gel was gently washed (1 minute) in distilled water, then 10 mL of the citrate-phosphate at pH 7.0 were added. After 2-3 minutes on an orbital shaker at 60 rpm, the buffer was decanted. Plates were scanned within 5-10 minutes using a Hewlett Packard Scan Jet 3c/T. The diameters of cleared areas were measured using MacRhizo™ (Regent Instruments Inc., Quebec, Canada) software (Appendix, Figure 1). Enzyme activity was calculated from standard curves using regression analysis (Apendix, Figure 2). Purified endo-β-mannanase (Megazyme, Ireland) was used as a standard.

Ethylene Determination

Three replications of 0.2 g of dry seeds were placed on two layers of 3.0 cm diameter germination paper (Anchor Paper, Hudson, WI) which were at the base of 38 mL volume vials sealed with rubber septa. The seeds in the vials were moistened with 3 mL of distilled water then incubated under the same conditions as the standard germination procedures. After 3, 6, 9, 12, 18, and 24 hours of imbibition, ethylene evolution was determined. In one study, ethylene was determined after 10 hours of imbibition (before radicle protrusion). One mL gas sample was withdrawn using a gas-tight hypodermic syringe. After sample withdrawal, the vials were flushed with air and sealed again for additional sampling. Ethylene was assayed using a gas chromatograph (Hewlett-Packard 5890 Series II) equipped with an alumina column and a flame ionization detector. The carrier gas was nitrogen and the column temperature was 100°C.

Analysis of Structural Polysaccharides

Lettuce seeds of DGB, EVE and PI were imbibed for 3 hours at 4°C to moisten the seed coat. Endosperms were excised by pressing the cotyledon end using the tip of a conical glass rod. The micropylar region was separated from the rest of the endosperm (lateral) using a surgical blade. Both were placed in ethanol (80%) and maintained at 4°C until analysis. Hydrolysis and acetylation (Jones and Albersheim, 1972) were used for the determination of cell wall noncellulosic neutral sugar composition. Neutral sugars were analyzed by a gas chromatograph (Hewlett-Packard 5890 Series II) equipped with a glass-capillary column (25 m x 0.2 mm x 0.33μm thickness, HP-5 crosslinked 5% Phenyl

Methyl Silicone), and a flame ionization detector. The carrier gas was helium and the column temperature was 250°C.

Experimental Design and Statistical Analysis

Ethylene evolution, enzyme activity, and germination tests were conducted using a randomized complete block design, using three replications per treatment. Germination percentages were transformed to a square root arc sine basis prior to statistical analysis. Analysis of variance (ANOVA) was performed by means of Statistical System (SAS) software (SAS, 1987). Treatment means were separated by the Duncan Multiple Range test. In analyzing the effect of light and temperature interaction, treatment means were separated by the Least Significant Difference (LSD) test. Correlation analyses were performed and Pearson correlation coefficients were generated using PROC CORR (SAS, 1987).

Results and Discussion

Germination was 96% or more for all of the genotypes incubated at 20°C (Table 3-1). The onset of seed germination (visible radicle protrusion in 50 % of the seeds) varied from 13 (PI) to 20 hours (DGB). At 20°C, no endo-β-mannanase activity was observed after 4 hours of imbibition (Table 3-1). Halmer (1989) observed endo-β-mannanase activity at 24°C in lettuce seeds only after 6 hours of seed imbibition, and suggested that endo-β-mannanase production was dependent on the completion of transcriptional events during the 6-hour lag phase. Enzyme activity in the present experiment was observed 1 hour before radicle protrusion in seeds from all genotypes except DGB (Table 3-1).

Table 3-1. Seed germination and endo-β-mannanase activity in the whole endosperm of lettuce genotypes imbibed in constant light at 20°C.[w]

Genotypes	Germination (%)	50 % radicle protrusion (h)	Mannanase activity (pmol min^{-1})[y]			
				Radicle protrusion		
			4 hours	1 hour before	1 hour after	24 hours
DGB	100 a	19 b	0	0.0 c	0.0 d	2 de
VAL	98 ab	20 b	0	0.8 bc	3.0 ab	12 d
FLO	96 b	16 ab	0	1.2 b	2.6 b	248 a
EVE	100 a	18 b	0	2.7 a	3.5 a	110 bc
PI	100 a	13 a	0	0.4 bc	1.5 c	152 b

[y] Enzyme activity was measured 1 hour before and 1 hour after radicle protrusion in each genotype. Also, after 4 and 24 hours of imbibition.

[w] Means separation by Duncan's Multiple Range test at $P \leq 0.05$.

Genotypes: 'Dark Green Boston' (DGB), 'Valmaine' (VAL), 'Floricos 83' (FLO), 'Everglades' (EVE), and PI 251245 (PI).

Halmer (1989) reported that endo-β-mannanase is synthesized *de novo*. Enzyme activity before radicle protrusion was sometimes higher in seeds incubated at 20°C (Table 3-1) than at 35°C (Table 3-2). This equated to observed endo-β-mannanase activity in the PI after 12 hours at 20°C and after 4 hours at 35°C. Similarly EVE germinated 8 hours earlier at 35°C compared to 20°C. Presumably, differences observed in endo-β-mannanase activity at 4 hours would account for faster germination of EVE or PI. Dutta et al. (1997) observed a suppression of endo-β-mannanase activity in 'Pacific' lettuce seeds at 32°C, while at 25°C endosperm cell walls exhibited active autolysis. Possibly at 35°C, protein synthesis is adversely affected or a factor(s) involved in the regulation of endo-β-mannanase production by the endosperm is inhibited in thermosensitive genotypes such as DGB.

At 35°C, only the two thermotolerant (EVE and PI) genotypes germinated above 90% (Table 3-2). At this temperature, seeds from the PI germinated after 6 hours, whereas seeds from FLO germinated after 16 hours (Table 3-1). DGB seeds germinated only 4%, whereas VAL did not germinate. Enzyme activity was not detected before radicle protrusion at 35°C in the thermosensitive DGB, VAL, and FLO genotypes (Table 3-2). This may be related to their inability to germinate at 35°C. However, a low (basal) amount of enzyme activity was detected prior to radicle protrusion at 35°C in the thermotolerant (EVE and PI) genotypes. Enzyme activity increased as time of imbibition increased, and in general, was higher in those genotypes that germinated earlier (Tables 3-1 and 3-2). The level of endosperm resistance during puncture tests with lettuce depended on genotype (Sung et al., 1998b). Using the same seed lots and genotypes as in

Table 3-2. Seed germination and endo-β-mannanase activity in the whole endosperm of lettuce genotypes imbibed in constant light at 35°C.[w]

Genotypes	Germination (%)	50 % radicle protrusion (h)	Mannanase activity (pmol min $^{-1}$)[y]			
				Radicle protrusion		
			4 hours	1 hour before	1 hour after	24 hours
DGB	4 c	14	0 b	0 b	0 c	2 d
VAL	0 c	-	0 b	0 b	-	0 d
FLO	26 b	16	0 b	0 b	13.5 a	255 a
EVE	96 a	10	1.1 a	1.2 a	2.7 b	25 c
PI	100 a	6	1.5 a	0 b	0 c	173 b

[y] Enzyme activity was measured 1 hour before and 1 hour after radicle protrusion in each genotype. Also, after 4 and 24 hours of imbibition.

[w] Means separation by Duncan's Multiple Range Test at $P \leq 0.05$.

Genotypes: 'Dark Green Boston' (DGB), 'Valmaine' (VAL), 'Floricos 83' (FLO), 'Everglades' (EVE), and PI 251245 (PI).

these experiments, Sung et al. (1998b) verified that thermotolerant genotypes had lower endosperm resistance than the thermosensitive types. Thermotolerant genotypes appeared to have weakened endosperm cell walls and depleted stored reserves (Sung, 1996; Sung et al., 1998a). Combining Sung's results with those presented in the present study, a relationship between seed germination at high temperature, lower resistance to endosperm rupture, and an increase in endo-β-mannanase activity before radicle protrusion was established.

In tomato, a positive relationship between germination ability at low temperature and endo-β-mannanase activity in six progeny lines was observed (Leviatov et al., 1995). The authors suggested that an increase in endo-β-mannanase activity in the micropylar region of the endosperm was essential for radicle protrusion, and the expression of enzyme activity at low temperature correlated with the cold-tolerant germinating lines.

Mannanase activity may also vary depending on the structure of the substrate. For example, increasing galactose in the galactomannan polymer reduced the activity of mannanase (McCleary and Matheson, 1975). With this in mind, a study was conducted to determine whether possible genotypic differences in germination at high temperature were related to endosperm sugar composition.

Significant differences in sugar make-up among the three genotypes used in this study were observed in both the micropylar and lateral regions of the endosperm (Figures 3-1, 3-2 and Tables 3-3 and 3-4). A higher amount of total sugars was observed in micropylar region than lateral region of the endosperm. Thermosensitive DGB had more galactose and mannose in the micropylar region compared to thermotolerant EVE and PI (Figure 3-1). High amounts of mannose and galactose in the cell wall could require more

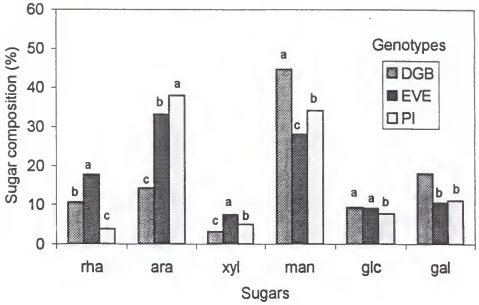

Figure 3-1. Percentage among six noncellulosic neutral sugars in the micropylar region of lettuce endosperm. Means separation within each sugar by Duncan's Multiple Range test at $P \leq 0.05$. Genotypes: 'Dark Green Boston' (DGB), 'Everglades' (EVE), and PI 251245 (PI).

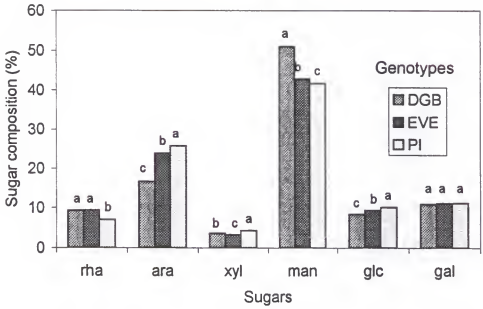

Figure 3-2. Percentage among six noncellulosic neutral sugars in the lateral region of lettuce endosperm. Means separation within each sugar by Duncan's Multiple Range test at $P \leq 0.05$. Genotypes: 'Dark Green Boston' (DGB), 'Everglades' (EVE), and PI 251245 (PI).

Table 3-3. Noncellulosic neutral sugars in micropylar and lateral region of endosperm tissue from different lettuce genotypes.

	Micropylar region			Lateral region		
	Genotype					
Sugar	DGB	EVE	PI	DGB	EVE	PI
	----- noncellulosic neutral sugar / endosperm tissue (µg / mg) -----					
Rhamnose	61.6 b[w]	91.4 a	19.9 c	27.6 a	23.8 b	13.3 c
Arabinose	81.7 c	170.2 b	192.4 a	48.9 b	60.5 a	48.9 b
Xylose	18.7 b	38.7 a	25.4 b	10.8 a	8.3 b	8.1 b
Mannose	254.3 a	144.2 b	172.7 b	151.5 a	108.6 b	79.4 c
Glucose	53.7 a	53.9 a	38.9 b	25.1 a	23.9 a	19.2 b
Galactose	101.5 a	54.8 b	56.7 b	32.5 a	28.4 b	21.3 c

[w]Means separation within each sugar and endosperm region by Duncan's Multiple Range test at $P \leq 0.05$. Genotypes: 'Dark Green Boston' (DGB), 'Everglades' (EVE), and PI 251245 (PI).

Table 3-4. Noncellulosic neutral sugar of micropylar and lateral endosperm tissue and galctose/mannose ratio from different lettuce genotypes.

	% of total noncellulosic neutral sugar of endosperm tissue (w/w)		Gal/Man ratio	
Genotypes	Micropylar	Lateral	Micropylar	Lateral
DGB	57.1 a	29.7 a[w]	0.40 b	0.21 a
EVE	51.7 a	25.4 b	0.37 ab	0.26 b
PI	51.1 a	19.0 c	0.33 a	0.27 c

[w] Means separation within each column by Duncan's Multiple Range test at $P \leq 0.05$.
Genotypes: 'Dark Green Boston' (DGB), 'Everglades' (EVE), and PI 251245 (PI).

time to complete hydrolysis by mannanase. In legume seeds, the extent of hydrolysis of galactomannans was governed by the galactose content of the polysaccharide (McCleary et al., 1976). It has been suggested that in developing legume seeds the action of α-galactosidase caused the low gal/man ratio of mature seeds by removing galactosyl residues from the galactomannan polymer (Kontos and Spyropoulos, 1996). Conversely, increasing galactose in the galactomannan polymer increases gal/man ratio and reduces the activity of mannanase.

Dutta et al. (1994) reported that during autolysis of lettuce endosperm cell wall, substantial amounts of arabinose and galactose were released in addition to mannose. From this study, it appears that cell wall-hydrolyzing enzymes other than endo-β-mannanase might be also involved in weakening of lettuce endosperm. In tomato, cell wall sugar composition affected enzyme activity in endosperm tissue (Dahal et al., 1997). Possibly, anatomical differences in the tomato endosperm region of the different genotypes could have contributed to different mannanase activity requirements (Dahal et al., 1997). For example, Hilhorst and Downie (1995) suggested that rapid germination of ABA-deficient tomato seeds was due to a thinner endosperm and seed coat. Further studies of the role of mannans (and mannanases) in lettuce seed germination should include isolating, purifying, and characterizing the target polymers. Immunocytochemistry using antibodies raised against purified lettuce seed galactomannan would also be of considerable use for monitoring the spatial and temporal path of wall hydrolysis preceding radicle emergence.

Sung et al. (1998a) reported that lettuce endosperm cell walls in front of the radicle from 'Dark Green Boston' and 'Everglades' (genotypes) seeds matured at high temperature were broken down more rapidly than those from seeds matured at low

temperatures. DGB seeds developed under high temperatures (30/20°C) germinated more at 20°C (Figure 3-3) than seeds developed under lower temperatures (20/10°C). EVE seeds developed either at 30/20°C or 20/10°C had similar germination (Figure 3-3). At 20°C, seeds from both genotypes developed under 20/10°C germinated after 23 hours and in seeds matured at 30/20°C after 21 hours.

In the present work, germination at 35°C of DGB produced at 20/10°C was 10%, whereas seeds produced at 30/20°C germinated 67 % (Figure 3-4). EVE seeds produced at 20/10°C and 30/20°C germinated at 35°C, 32 and 83%, respectively (Figure 3-4). Seeds from both genotypes produced at 20/10°C took about 20 more hours to germinate than seeds matured at 30/20°C. Thus, seeds matured at high temperature germinate better regardless of genotype.

Higher enzyme activity was observed 1 hour before radicle protrusion in DGB seeds produced under 30/20°C compared with those produced at 20/10°C. This was especially true when seeds were germinated at 35°C (Figure 3-4). Enzyme activity was not detected before radicle protrusion in seeds from DGB developed at 20/10°C (10% of germination). Thus, seed maturation at high temperature partially overcame the inhibitory effect of high temperature on DGB germination, possibly due to the observed increase in endo-β-mannanase activity during germination.

Temperature during seed maturation and development affects seed chemical composition, such as oil quality or protein concentration (Fenner, 1992; Miquel and Browse, 1995). Gray et al. (1988) suggested that the synthesis of cell-wall-weakening enzymes should be activated during seed development and maturation. Although enzyme

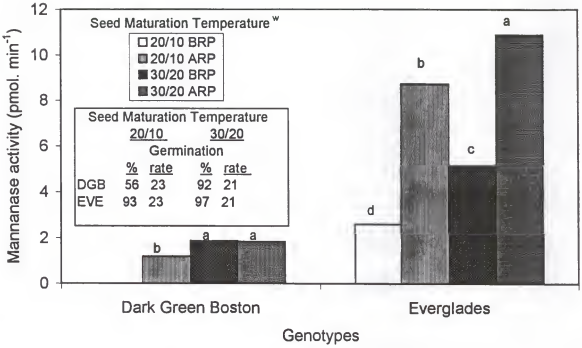

Figure 3-3. Germination and endo-β-mannanase activity of whole lettuce endosperm at 20°C of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) seeds developed at two different day/night temperature regimes. ᵂBRP = 1 hour before radicle protrusion; ARP = 1 hour after radicle protrusion. Means separation within genotype by Duncan's Multiple Range test at $P \leq 0.05$.

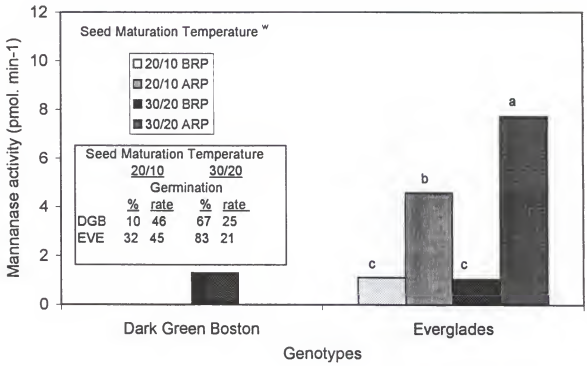

Figure 3-4. Germination and endo-β-mannanase activity at 35°C of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) seeds developed at two different day/night temperature regimes. ᵂBRP = 1 hour before radicle protrusion; ARP = 1 hour after radicle protrusion. Means separation within genotype by Duncan's Multiple Range test at $P \leq 0.05$.

activity was not assayed in dry seeds used in the present study, Hilhorst and Downie (1995) observed early activity of mannanase in tomato, and suggested that this was residual activity from seed development.

High temperatures inhibit ethylene production and exogenous ethylene overcomes the inhibitory effect of high temperature on lettuce seeds (Abeles, 1986; Khan and Prusinski, 1989; Saini et al., 1989). Thus, ethylene evolution during germination was determined in order to verify the possible differences in ethylene production among the thermosensitive and thermotolerant genotypes. At 20°C, ethylene production was not detected before 6 hours of imbibition (Figure 3-5). Ethylene was detected from germinating seeds of FLO and PI 6 and 9 hours respectively, from the initiation of imbibition. After 9 hours from the initiation of imbibition, ethylene production was observed in all genotypes, except DGB, which also produced the lowest amount of ethylene during the 24-hour imbibition period. The highest amount of ethylene was detected after radicle protrusion in all the genotypes, regardless of temperature. Khan (1994) reported that little or no ethylene was produced in lettuce before germination, but relatively large amounts of ethylene were produced at the time of radicle. Perhaps the low amount of ethylene observed prior to radicle protrusion was because ethylene was "trapped" beneath the integuments of the seed, and during radicle protrusion, the rupture of endosperm and seed coat allowed free ethylene to be "released".

At 35°C, the first detectable ethylene production occurred between 9 and 12 hours in FLO, EVE, and PI (Figure 3-6). Moreover, thermotolerant genotypes produced more ethylene during seed germination at high temperature than the thermosensitive genotypes. Conversely, seeds from thermosensitive DGB and VAL produced the least amount of

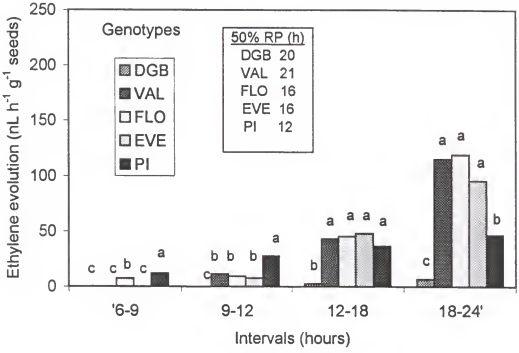

Figure 3-5. Ethylene evolution in lettuce seeds incubated in light at 20°C. Genotypes: 'Dark Green Boston' (DGB), 'Valmaine' (VAL), 'Floricos 83' (FLO), 'Everglades' (EVE), and PI 251245 (PI). RP (radicle protrusion). Means separation within each interval by Duncan's Multiple Range test at $P \leq 0.05$.

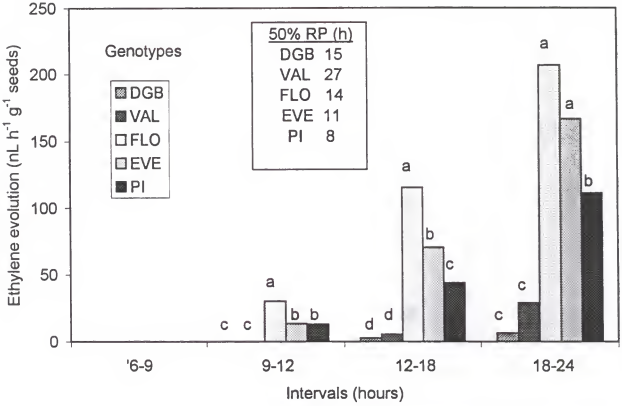

Figure 3-6. Ethylene evolution in lettuce seeds incubated in light at 35°C. Genotypes: 'Dark Green Boston' (DGB), 'Valmaine' (VAL), 'Floricos 83' (FLO), 'Everglades' (EVE), and PI 251245 (PI). RP (radicle protrusion). Means separation within each interval by Duncan's Multiple Range test at $P \leq 0.05$.

ethylene at 35°C. These results agree with Prusinski and Khan (1990), who reported that the ability of lettuce genotypes to produce ethylene during high temperature stress corresponded with their ability to germinate. They suggested this as a criterion to select thermotolerant lettuce cultivars. The thermotolerant genotypes that produced more ethylene at high temperature (Figure 3-6) also produced more endo-β-mannanase and germinated earlier (Table 3-2). Thus, an association between ethylene evolution, endo-β-mannanase activity prior to radicle protrusion, and seed germination at high temperature was verified.

At high temperature, lettuce seed is extremely temperature sensitive and requires red light in order to germinate maximally (Evenari et al., 1953; Vidaver and Hsiao, 1974; Heydecker and Joshua, 1977; Blaauw-Jasen, 1981; Georghiou and Thanos, 1983; Saini et al., 1989). EVE, a thermotolerant lettuce genotype, germinated above 90% at temperatures up to 35°C in light (Guzman et al., 1992). However, germination of EVE decreased when seeds were imbibed under high temperature in the dark (Sung, 1996). Thus, there is an important interaction between light and high temperature, which may mediated by the phytochrome system (Taylerson and Hendriks, 1972; Fielding et al., 1992).

The regulation of germination at high temperature by ethylene is also enhanced in light (Dunlap and Morgan, 1977), suggesting that the process may also be mediated by phytochrome (Negm and Smith, 1978). In addition, high temperature (35 to 40°C) may inhibit ethylene production in various plant tissues (Yu et al., 1980). Thus, another study was conducted to investigate the possible correlation between endo-β-mannanase activity, ethylene production and germination under light and dark conditions at

three temperatures in EVE lettuce seed. A significant interaction for all the above parameters was observed (Table 3-5). At 20°C, seeds imbibed in either light or dark germinated 100% after 16 hours. At 27.5°C, seeds germinated after 13 hours, and germination was 100 and 92% in light and dark, respectively. At 35°C, seeds imbibed in light germinated 94 %, whereas those in dark germinated only 7%; both began germinating at 13 hours. Before radicle protrusion, endo-β-mannanase activity assayed in the micropylar endosperm region was higher at 20°C in dark or at 27.5°C in light (Table 3-5). With increasing temperature during imbibition in dark, enzyme activity decreased in the micropylar endosperm region prior to radicle protrusion. At 35°C in dark, no enzyme activity was detected before radicle protrusion.

Endo-β-mannanase activity was also observed in the lateral endosperm prior to radicle protrusion and increased markedly after radicle protrusion (Table 3-5). Bewley and Halmer (1980/81) reported that lettuce seeds imbibed in light produced high amounts of endo-β-mannanase, but this was only observed after radicle protrusion. Seeds imbibed in the dark produced little endo-β-mannanase (Halmer et al., 1976), but that was not true for this study at 27.5°C. Thus, conditions where phytochrome-induced lettuce seed germination was inhibited also lead to a reduction in endo-β-mannanase activity. For example, at 35°C in the dark, germination was 7%, and essentially no enzyme activity could be detected prior to radicle protrusion. Incubation of 'Pacific' lettuce seeds in the dark at 32°C resulted in no germination and almost complete suppression of endo-β-mannanase (Dutta et al., 1997). Endo-β-mannanase activity before radicle protrusion in the light was lower at 35 compared to 27.5°C (Table 3-5).

Table 3-5. Ethylene production, mannanase activity, and germination of 'Everglades' lettuce seeds incubated at different conditions.

Temp (°C)		Germ (%)	Mannanase activity (pmol min $^{-1}$) Micropylar Region Before rad. protrusion	Micropylar Region After rad. protrusion	Lateral Region Before rad. protrusion	Lateral Region After rad. protrusion	Ethylene (pL h^{-1} g^{-1} seeds)[y]
20	Light	100	1.2	2.2	1.1	61.0	934
	Dark	100	1.6	2.2	1.2	28.0	896
27.5	Light	100	1.4	6.4	3.8	117.3	1424
	Dark	92	1.2	6.2	1.1	155.1	1232
35	Light	94	1.2	1.6	1.1	15.4	1066
	Dark	7	0.0	2.7	0.0	9.8	0
Significance							
Temp (T)		** [w]	**	**	**	**	**
Light (L)		**	**	**	**	**	**
T x L		**	**	**	**	**	**
LSD (0.05)		5.6	0.14	0.18	0.23	0.14	64

[y]Ethylene was determined after 10 hours of imbibition (before radicle protrusion)
[w] Significant at $P \leq 0.01$ by F-test.

Ethylene produced by seeds in the light was slightly more than in the dark at each temperature. Saini et al. (1989) reported that in 'Grand Rapids' lettuce, ethylene evolution in red light-incubated seeds began to increase 2 hours prior to radicle protrusion, whereas the dark-incubated seeds produced a low and constant amount of ethylene at 32°C. Ethylene production during seed germination at 35°C was lower than at 27.5°C, particularly in the dark. The optimum temperature for ethylene production in some fruit tissue is near 30°C, and starts to decline in temperatures above 30°C until it ceases near 40°C (Abeles et al., 1992). In lettuce, a decrease in ethylene synthesis was also observed under high temperature during seed imbibition (Burdett, 1972b; Dunlap and Morgan, 1977; Abeles, 1986; Khan and Huang, 1988). In the present study, ethylene production was not observed at 35°C in the dark after 10 hours of imbibition. The ethylene produced at 10 hours (e.g., before radicle protrusion) correlated with enzyme activity in the micropylar region prior to radicle protrusion ($r = 0.85$), and with germination ($r = 0.90$). Consequently, a high correlation ($r = 0.96$) was observed between enzyme activity in the micropylar region prior to radicle protrusion and seed germination.

Are ethylene and mannanase two factors that might regulate the thermotolerance character in lettuce seeds? It was observed that higher endo-β-mannanase activity before radicle protrusion and ethylene evolution in seeds from thermotolerant genotypes occurred in this work. Also, regardless of genotype, seeds matured at high temperature produced more enzyme activity, and germinated better at high temperature than those matured at low temperature. Conditions that inhibited seed germination, such as high temperature in thermosensitive genotypes, or high temperature under dark conditions in thermotolerant genotypes also had reduced endo-β-mannanase activity and ethylene production. The

puncture tests and the anatomical studies using these same lettuce genotypes (Sung, 1996; Sung et al., 1998b) give further evidence that the weakening of the endosperm tissue around the radicle tip prior to radicle protrusion was related to the regulation of lettuce germination at high temperature. Thus, genotype and seed maturation temperature could overcome high temperature inhibition by increased endo-β-mannanase activity and/or ethylene production. A relationship between seed germination at high temperature and an increase of ethylene evolution and endo-β-mannanase activity before radicle protrusion was established in this study. Although there is evidence that ethylene might be involved in this mechanism, further investigations are needed to determine its actual role during germination.

Summary

Continuous high temperature can lead to a reduction or complete inhibition of lettuce seed germination. Weakening of the endosperm tissue around the radicle tip prior to radicle protrusion and the role of hydrolytic enzymes in endosperm have been associated with normal germination in this species. The galactomannan polysaccharides in the lettuce endosperm cell wall are mobilized by endo-β-mannanase. The role of endo-β-mannanase during germination of lettuce seeds at high temperature (35°C) was investigated by gel-diffusion assay utilizing thermotolerant and thermosensitive lettuce genotypes, seeds produced under low and high temperature regimes, and seeds incubated at non-inhibitory and inhibitory conditions for germination. Ethylene production was measured during seed germination in those genotypes. Seeds from thermotolerant ('Everglades' and PI 251245) genotypes had higher endo-β-mannanase activity before

radicle protrusion at 35°C than thermosensitive ('Dark Green Boston', 'Valmaine' and 'Floricos 83') genotypes. Significant differences in endosperm sugar composition were also observed among the genotypes. Higher amount of total sugars was observed in micropylar compared to lateral region of the endosperm. Genotypes with high ethylene production at high temperature also had higher enzyme activity. At 35°C, germination of 'Dark Green Boston' and 'Everglades' seeds produced at a 20/10°C day/night temperature regime was 10 and 32%, respectively, whereas seeds produced at 30/20°C day/night temperature regime germinated 67 and 83%, respectively. Higher enzyme activity was observed before radicle protrusion in 'Dark Green Boston' seeds produced under 30/20°C compared with those produced at 20/10°C. A relationship between seed germination at high temperature, ethylene production, and an increase in endo-β-mannanase activity before radicle protrusion was verified.

CHAPTER 4
ETHYLENE EVOLUTION AND ENDO-β-MANNANASE ACTIVITY DURING LETTUCE SEED GERMINATION AT HIGH TEMPERATURE

Introduction

High temperatures during lettuce seed imbibition can delay or completely inhibit germination. The lettuce seed embryo is completely enclosed within an endosperm where the radicle must penetrate in order to grow and the seed to germinate. The lettuce endosperm layer appears to restrict radicle protrusion, especially at high temperature (Sung et al., 1998a). Ikuma and Thimann (1963) proposed that the action of an enzyme produced by the embryo enabled the radicle tip to penetrate through the restricting tissues. They did not isolate or identify the enzyme. Recently, Dutta et al. (1997) reported that a cell-wall-bound endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion and was regulated by the same conditions that govern seed germination. These authors suggested that endo-β-mannanase was involved in weakening of the lettuce endosperm cell walls.

Ethylene overcomes the inhibitory effect of high temperature on lettuce seed germination (Abeles, 1986; Abeles and Lonski, 1969; Burdett, 1972a; Dunlap and Morgan, 1977; Fu and Yang, 1983; Huang and Khan, 1992; Keys et al., 1975; Khan and Prusinski, 1989; Negm et al., 1972; Rao et al., 1975; Saini et al., 1986; Saini et al., 1989). However, the mechanism of ethylene action during seed germination is not understood.

High temperatures (35 to 40°C) inhibited ethylene production in various plant tissues, such as apple and mung bean (Yu et al., 1980). Burdett (1972b) pretreated 'Grand Rapids' lettuce seeds at 30°C for 36 hours and then imbibed the seeds at 20°C. They reported that the pretreatment at high temperature reduced germination primarily through its inhibitory effect on ethylene production by the seeds.

Several authors reported that ethylene synthesis or sensitivity to ethylene action in lettuce was decreased during seed imbibition at high temperature (Abeles, 1986; Burdett, 1972a; Dunlap and Morgan, 1977; Khan and Huang, 1988). Abeles (1986) reported that exogenous ethylene overcame thermoinhibition at 30°C but not at 33°C in dark. Similar results were also reported by Dunlap and Morgan (1977) where exogenous ethylene promoted germination at 32°C but not at 36°C. Thus, high temperature stress may inhibit ethylene production and/or action and raise the threshold concentration of ethylene needed for lettuce seed germination. Abeles and Lonski (1969) however, could not increase germination of lettuce seeds with exogenous ethylene, once the seeds were dormant due to high temperature. They could however, stimulate lettuce seeds to germinate at high temperature before they become thermodormant. Burdett (1972a) reported an increase in ethylene production in lettuce seeds as temperature increased from 20 to 30°C; thus ethylene production was not thought to be limiting factor for germination at temperatures up to 30°C.

Another critical factor in ethylene studies is the use of inhibitors of ethylene synthesis or action to study germination. Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, generally had little influence on lettuce seed germination. For example, germination at 25°C (Huang and Khan, 1992) or 35°C (Khan and Prusinski, 1989) was

not inhibited by AVG even though ethylene production was. However, Abeles (1986) reported a reduction in lettuce germination of 50% or more with AVG at 25 and 30°C; the inhibition was overcome by the addition of ethylene.

Ethylene appears to have a "softening effect" on lettuce endosperm tissue (Abeles, 1986). Lettuce endoperms were incubated in air or in 10 μL/L of ethylene and mechanical force required to penetrate the endosperm was measured by puncture test; a lesser force was observed in seeds incubated in ethylene compared to air (Abeles, 1986). This action was not correlated with its effect as a germination promoter, since the effect was only observed at 20 but not at 30°C. Abeles (1986) also reported that the action of ethylene on lettuce seed germination was the promotion of radicle cell expansion in the embryonic hypocotyl; as the radicle cells expanded they pushed through the endosperm and seed coat.

Dutta and Bradford (1994) reported that ACC (via conversion to ethylene) extended the high temperature limit for lettuce seed germination by acting in the embryo to maintain a lower water potential threshold for the initiation of growth as temperature was increased. By doing, so the embryo could grow and push through the endosperm and seed coat. Huang and Khan (1992) stated that improved performance of primed lettuce seeds at high temperature was related to high vigor and greater capacity to produce ethylene. The authors reported that increasing priming duration with moist Micro-Cel E at 15°C increased ACC production and germination at 35°C. Thus, the involvement of ethylene in lettuce seed germination, particularly at high temperature, is still not defined.

Ethylene has also been reported to stimulate the synthesis of some enzymes in various species (Cervantes et al., 1994; Hasegawa et al., 1995). Separation of pepper and

peach cells in the abscission zones due to the activation of cell wall enzymes, such as endo-β-1,4-glucanases was reported as a ethylene effect (Casadoro et al., 1998). In melon fruit, other cell wall-degrading enzymes were classified as ethylene-dependent, such as endopolygalacturonase, some isoforms of β-galactosidase, α-arabinosidase, and galactanase (Pech et al., 1998). The climacteric ethylene rise in apple and pear fruits was accompanied by an increase in α-galactosidase and α-mannosidase (Moya et al., 1998).

Based on previous studies and assuming that weakening of the endosperm must occur for lettuce seed to germinate at high temperature (Chapter 3), ethylene might overcome the inhibitory effect of high temperature by activating a cell wall enzyme responsible for endosperm digestion, possibly the same effect of cell wall enzymes suggested 35 years ago by Ikuma and Thiman (1963). The objective of the present study was to determine the association of ethylene with endo-β-mannanase activity on lettuce seed germination at high temperature.

Materials and Methods

Plant Material

Lettuce (*Lactuca sativa* L.) seeds (achenes) from thermosensitive 'Dark Green Boston' (DGB) and thermotolerant 'Everglades' (EVE) genotypes were used in this study. Thermotolerance was defined as the ability of seeds to germinate above 90% at temperatures up to 35°C in light (Guzman et al., 1992; Sung, 1996). DBG and EVE were chosen because the genetic relation between these two genotypes (Guzman et al., 1992). All seeds were produced in the same season and region of San Joaquin Valley, California, in 1994. Seeds were stored at 10°C, 40% RH until used.

Seed Priming

Seeds were also primed in 200 mm test tubes for three days at 15°C with constant light (~26 μmol m^{-2} s^{-1}) in an aerated solution of polyethylene glycol (PEG), osmotic potential of -1.2 MPa), or PEG + 10 mM of 1-aminocyclopropane-1-carboxylic acid (ACC), or PEG + 10 mM of aminoethoxyvinylglycine (AVG), or PEG + 20 mM of silver thiosulphate (STS) (30 mL of solution g^{-1} of seed). An aquarium pump provided aeration. The air was pre-hydrated by bubbling through water to minimize evaporation from the priming solution. Afterward, seeds were placed in a Buchner funnel, rinsed three times with 100 mL of distilled water, and redried in an incubator at 15°C and 45% RH for three days.

Seed Moisture Content

Seed moisture content was determined after drying the seeds for 1 hour at 130°C. The seeds were then placed in a desiccator at room temperature for 20 minutes before weighing (AOSA, 1993). Samples were replicated twice.

Seed Germination

Four replications of 25 seeds were placed on two layers of 5 cm diameter blotter paper (Anchor Paper, Hudson, WI) moistened with 3 mL of distilled water. Distilled water was added as needed to keep the filter paper moist. Blotters were covered with 5.5 cm petri dishes lids and incubated at 20 or 35°C under constant light (~26 μmol m^{-2} s^{-1}) on a one-dimensional thermogradient bar (Type DB 5000, Van Dok & De Boer, B.V., Holland). Seeds were also germinated in ACC (10 mM), AVG (10 mM), and STS (20 mM) solutions. Germinated seeds were counted daily at the same time. Germination rate

was calculated according to the formula Σ Ti Ni / Σ Ni, where Ni is the number of newly germinated seed at time Ti (Maguire, 1962).

Enzyme Activity

A gel-diffusion assay (Downie et al., 1994; Still et al., 1997) was used to measure endo-β-mannanase (EC 3.2.1.78) activity after seed priming and during seed germination. The methodology is described in detail in Chapter 3.

Ethylene Determination

Ethylene was measured as described in Chapter 3.

Experimental Design and Statistical Analysis

Ethylene evolution, enzyme activity and germination tests were conducted using a randomized complete block design, using three replications per treatment. Germination percentages were transformed to a square root arc sine basis prior to statistical analysis. Analysis of variance (ANOVA) of data was performed by means of Statistical System (SAS) software (SAS, 1987). Treatment means were separated by the Duncan Multiple Range test.

Results and Discussion

In thermosensitive DGB, STS reduced both germination and germination rate in light at 20°C (Table 4-1). DGB germinated only 33% in water at 35°C, whereas incubation in ACC solution increased seed germination to 92% (Table 4-1). Seeds with ACC began to germinate after 12 hours whereas in water and AVG treatments, germination began after 16 hours. At 35°C, STS completely inhibited germination of DGB seeds, but AVG did not affect seed germination. ACC, the immediate precursor of

Table 4-1. Seed germination of lettuce 'Dark Green Boston' (DGB) and 'Everglades' (EVE) incubated in different solutions in light at 20 and 35°C.

Treatments	Genotype							
	DGB				EVE			
	Germination Temperature (°C)							
	20		35		20		35	
	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)
Control	98 a[z]	24 a	33 b	43 a	100 a	24 a	98 a	24 a
ACC	100 a	24 a	92 a	38 a	100 a	24 a	100 a	24 a
AVG	100 a	24 a	48 b	43 a	100 a	24 a	98 a	24 a
STS	77 b	48 b	0 c	NA[y]	100 a	25 a	49 b	24 b

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

[y] No germination.

ethylene biosynthesis (Yang and Hoffman, 1984), has been implicated in the alleviation of high temperature on lettuce seed germination (Abeles, 1986; Saini et al., 1986; Khan and Prusinski, 1989; Huang and Khan, 1992). STS, a putative specific inhibitor of ethylene action, interacts with the binding site for ethylene (Beyer, 1976; Veen, 1983); the copper ion may be involved (Knee, 1995). STS decreased seed germination of DGB at both temperatures, and EVE at 35°C. This suggests that ethylene is in fact involved in lettuce seed germination at high temperature and is specifically needed for germination of thermosensitive genotypes.

Abeles (1986) reported that 'Grand Rapids' lettuce seeds germinated approximately 25% at 30°C in the dark, but only 10% germinated in AVG. Huang and Khan (1992) reported a reduction of 50 % in germination using AVG during 'Mesa 659' lettuce germination at 35°C in the light, and the inhibitory effect of AVG was completely reversed by adding ACC. When nontreated lettuce seeds were imbibed at 35°C, no ACC synthesis was detected.

At 20°C, thermotolerant EVE germinated 100%, even in the presence of AVG and STS (Table 4-1). At 35°C, control seeds of EVE germinated 98% after 12 hours but STS reduced germination to 49%. Germination rate was reduced by STS. Germination in AVG or ACC was the same as the control. Abeles (1986) also found an inhibitory effect of 50% of STS on seeds germinated at 30°C compared to those germinated at 25°C.

ACC synthase, which converts S-adenosylmethionine (SAM) to ACC, is an important site of control of ethylene biosynthesis (Yang, 1980). ACC synthase, a pyroxidal enzyme, is inhibited by AVG, which is an inhibitor of all pyridoxal phosphate-dependent enzymes (Yu et al., 1979). In the present study, AVG did not inhibit lettuce

germination possibly because endogenous levels of ACC were converted to ethylene in the light at high enough levels for germination to occur. Moreover, the concentration of ethylene needed for action was maintained within the embryonic tissues encased by the endosperm and seed coat might have been sufficient to allow seed germination at 20 and 35°C in lettuce even when AVG was added.

In untreated seeds of DGB at 20°C, ethylene evolution was first observed during the 9-12 hour collection period (Table 4-2). At 35°C, ethylene was detected only during the 12-24 hour collection period. Adding ACC lead to early detection of ethylene in either genotype at either temperature. AVG completely inhibited ethylene at both temperatures in both genotypes (Tables 4-2 and 4-3). STS delayed ethylene evolution at both temperatures in both genotypes. Ethylene was detected in EVE during the 9-12 hour imbibition time at either 20 or 35°C (Table 4-3). Previously, EVE was shown to have the ability to produce ethylene during seed germination, even at high temperature (Chapter 3). Prusinski and Khan (1990) reported that the ability of lettuce genotypes to produce ethylene during high temperature stress generally corresponded with their ability to germinate.

In the present study, ethylene evolution was not detected under the limits of the experimental procedures when AVG was applied, although germination did occur even at 35°C. The significance of this finding is not clearly understood, however potentially either treshold levels for ethylene evolution/action were not detectable (possibly due to restriction of ethylene from the seed coverings, production at lower levels than detectable, or immediate utilization) or ethylene was not totally necessary for the induction of endo-β-mannanase at 35°C (i.e., there is an alternate pathway).

Table 4-2. Ethylene evolution during 'Dark Green Boston' seed germination in light at 20 and 35°C.

	Germination Temperature (°C)							
	20				35			
	Time of Ethylene Collection (hours)							
Treatments	0-6	6-9	9-12	12-24	0-6	6-9	9-12	12-24
	Ethylene Concentration (nL h^{-1} g^{-1} seed)							
Control	0 b[z]	0 b	25 b	64 b	0 b	0 b	0 b	5 b
ACC	21a	168 a	215 a	542 a	108 a	179 a	250 a	579 a
AVG	0 b	0 b	0 c	0 c	0 b	0 b	0 b	0 b
STS	0 b	0 b	0 c	18 c	0 b	0 b	0 b	2 b

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

Table 4-3. Ethylene evolution during 'Everglades' seed germination in light at 20 and 35°C.

	Germination Temperature (°C)							
	20				35			
	Time of Ethylene Collection (hours)							
Treatments	0-6	6-9	9-12	12-24	0-6	6-9	9-12	12-24
	Ethylene Concentration (nL h^{-1} g^{-1} seed)							
Control	0	0 b[z]	27 b	100 b	0 b	0 b	8 b	59 b
ACC	0	121a	253 a	2048 a	187 a	360 a	643 a	1716 a
AVG	0	0 b	0 b	0 c	0 b	0 b	0 b	0 c
STS	0	0 b	0 b	40 c	0 b	0 b	0 b	3 c

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

Ethylene production increased as time of imbibition increased (Tables 4-2 and 4-3). Ethylene evolution prior to radicle protrusion was low and not detected before the 9-12 hour collection period in seeds imbibed in water or with ethylene inhibitors (Tables 4-2 and 4-3). Several authors (Fu and Yang, 1983; Saini et al., 1986; Khan, 1994) reported that little or no ethylene was produced before radicle protrusion. In the present study, little or no ethylene was detected in seeds at 16 and 12 hours at 20 and 35°C, respectively, and relatively large amounts of ethylene were produced at or after radicle protrusion. Perhaps the low amount of ethylene observed prior to radicle protrusion was because ethylene could be "trapped" within the seed via the seed coverings. During radicle protrusion the rupture of endosperm and seed coat might allow trapped ethylene "release". Also gas exchange may be impeded by the seed coverings, thus reducing ethylene production. Prusinski and Khan (1990) reported that the lettuce seed coverings might create a hypoxic environment unfavorable for the conversion of ACC to ethylene.

As previously mentioned, ethylene has been reported to regulate synthesis of cell wall enzymes (Casadoro et al., 1998; Moya et al., 1998; Pech et al., 1998). Endo-β-mannanase was involved in weakening of the lettuce endosperm cell walls at high temperatures (Dutta et al., 1997; Chapter 3). Thus, endo-β-mannanase activity was assayed during seed germination at 35°C (Table 4-4). More endo-β-mannanase activity was detected prior to radicle protrusion in seeds from thermotolerant EVE than thermosensitive DGB. No endo-β-mannanase activity was detected 1 hour before radicle protrusion when seeds were imbibed in AVG or STS in DGB (Table 4-4). Perhaps, the amount of endo-β-mannanase activity in those seeds was not enough to be detected by the gel-diffusion assay used in the present study. Conversely, adding ACC during imbibition

Table 4-4. Endo-β-mannanase activity at 1 hour before radicle protrusion of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) lettuce seeds germinated in light at 35°C in water, ACC (10 mM), AVG (10mM), and STS (20 mM) solutions.

	Genotype	
	DGB	EVE
Treatments	Endo-β-mannanase activity (pmol min^{-1})	
Control	0.0 b[z]	1.1 b
ACC	1.0 a	1.7 a
AVG	0.0 b	1.0 b
STS	0.0 b	0.0 c

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

lead to increased endo-β-mannanase activity over the control. In EVE, endo-β-mannanase activity at 35°C was associated with 98% germination in the control and 100% germination when ACC was added. Lack of endo-β-mannanase activity 1 hour before radicle protrusion in thermosensitive DGB was associated with 33% germination in the control. When ACC was added and endo-β-mannanase activity was detected, 92% germination was recorded. When STS was added, little or no endo-β-mannanase activity was detected and little or no germination ensued at 35°C in both genotypes.

Seed priming overcomes thermoinhibition and/or thermodormancy of lettuce seeds (Guedes and Cantliffe, 1980; Cantliffe et al., 1981; Khan et al., 1980/81). The mechanism of seed priming in thermosensitive lettuce was suggested to be due to the weakening of endosperm because of increased endo-β-mannanase activity (Nascimento et al., 1998a; Nascimento et al., 1998b; Chapter 5). Thus, a study was conducted to investigate whether ethylene could be involved in the weakening of the endosperm. Seeds were primed in solutions of PEG, or PEG + ACC (10 mM), or PEG + AVG (10 mM), or PEG + STS (20 mM).

The original seed moisture content (SMC) of DGB and EVE before priming was 5.9 and 5.6%, respectively. After drying, SMC varied from 4.7 to 6.5%. In DGB, differences in SMC were observed among priming treatments after soaking (Appendix, Table 1) but probably there was no practical significance to these findings. No radicle protrusion occurred during the priming treatment. During priming, seeds of both genotypes attained approximately a 40% water content after 48 hours. Normally these seeds would attain over 100% increase in fresh weight if soaked in water only.

Adding ACC to the priming solution had no significant effect on germination, but

the seeds from both genotypes germinated slightly faster than those primed in PEG solutions (Table 4-5). Seed germination was decreased by priming in AVG for both genotypes. Huang and Khan (1992) verified an inhibition of seed germination when AVG was applied during either priming or imbibition but AVG applied only during priming had less of an inhibitory effect in lettuce seed germination presumably due to some absorbency by osmotic solution (Micro-Cel E). They also found more ACC content during germination at 35°C in primed seeds compared to nonprimed seeds. In addition, the ability of primed lettuce seed to germinate at high temperature was related to high vigor and greater capacity to produce ethylene (Huang and Khan, 1992).

Priming with STS reduced germination of both genotypes (Table 4-5). In DGB, priming in PEG + STS completely inhibited subsequent germination at 35°C, but reduced germination in EVE. Thus, the beneficial effects of seed priming in alleviating thermoinhibition in thermosensitive lettuce genotypes could be negated by the absence of ethylene action. AVG and STS treatments during seed priming led to reduced endo-β-mannanase activity before radicle protrusion in both genotypes (Table 4-6). Thus, an increase of endo-β-mannanase caused by priming was negated by STS. Higher enzyme activity was observed in seeds primed with ACC compared to AVG or STS. An association between ethylene evolution, endo-β-mannanase activity and seed germination at high temperature was observed.

In order to conclude the exact role of ethylene in lettuce seed germination at high temperature, further studies are needed. The use of other specific inhibitors of ethylene action might be a good approach. 1-Methylcyclopropane (1-MCP), a gas releasing compound, is a competitive inhibitor of ethylene action, which binds to the ethylene

Table 4-5. Seed germination of lettuce 'Dark Green Boston' (DGB) and 'Everglades' (EVE) after priming seed dry back and reimbibition in water in light at 35°C.

Priming	Genotype			
	DGB		EVE	
	Imbibition Time (h)			
	5	24	5	24
PEG	40 ab[y]	99 a	49 a	99 a
PEG + ACC	56 a	100 a	50 a	100 a
PEG + AVG	26 b	93 b	0 b	91 b
PEG + STS	0 c	0 c	0 b	96 a

[z]Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

Table 4-6. Endo-β-mannanase activity at 1 hour before radicle protrusion of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) lettuce seeds primed in PEG solutions, PEG + ACC (10mM), PEG + AVG (10mM), and PEG + STS (20mM) and, after dry back, germinated in light at 35°C in water.

	Genotype	
	DGB	EVE
Treatments	Endo-β-mannanase activity (pmol min^{-1})	
PEG	1.3 b[z]	81 ab
PEG + ACC	2.1 a	100 a
PEG + AVG	1.1 b	4.8 c
PEG + STS	0.0 c	0.0 d

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

receptor to regulate tissue responses to ethylene (Tian, 1998). In addition, the use of transgenic plants and/or mutants could facilitate the elucidation of the mechanisms of ethylene biosynthesis as well as the role of ethylene in seed germination. Even so, evidence found in this study suggested that endo-β-mannanase might be regulated by ethylene, and that increased endo-β-mannanase activity before radicle protrusion might contribute to lettuce endosperm weakening, leading to a germination at high temperature. Sung et al. (1998b) reported that seeds from thermotolerant genotypes required less force to penetrate the lettuce endosperm than did thermosensitive genotypes and suggested that weakening of the endosperm layer of lettuce seeds was a pre-requisite to radicle protrusion at high temperatures. These findings correlated directly with the findings for control of endo-β-mannanase activity.

Summary

The role of endo-β-mannanase during lettuce seed germination at 35°C and the influence of ethylene in endo-β-mannanase regulation were investigated. Seeds of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) were germinated in water, or 10 mM of 1-aminocyclopropane-1-carboxylic acid (ACC), or 10 mM of aminoethoxyvinylglycine (AVG), or 20 mM of silver thiosulphate (STS). Seeds were also primed in polyethylene glycol (PEG), or PEG + ACC, PEG + AVG, or PEG+STS. Untreated seeds germinated 100% at 20°C. At 35°C, EVE germinated 100%, whereas DGB germinated only 33%. Seed priming or adding ACC during incubation increased germination at 35°C. Aminoethoxyvinylglycine (AVG) did not inhibit seed germination of DGB at 35°C, but STS did. Higher enzyme activity was observed in EVE compared with DGB seeds.

Providing ACC either during priming or during germination increased endo-β-mannanase activity, whereas AVG and STS lead to decrease or no activity. Higher ethylene evolution was detected in EVE than DGB during germination at 35°C. Silver thiosulphate (STS) reduced seed germination of both genotypes at high temperature. The results suggest that ethylene may overcome the inhibitory effect of high temperature in thermosensitive lettuce seeds due to increased endo-β-mannanase possibly leading to a weakening of endosperm.

CHAPTER 5
ENDO-β-MANNANASE ACTIVITY AND SEED GERMINATION OF THERMOSENSITIVE AND THERMOTOLERANT LETTUCE GENOTYPES IN RESPONSE TO SEED PRIMING

Introduction

Conditions of high temperature during sowing either in the greenhouse (transplant industry) or field can cause lettuce seed germination to be erratic or completely inhibited. Depending upon the genotype, lettuce seeds imbibed at temperatures above 25°C many times fail to germinate (Gray, 1975). This can lead to two different phenomena. The first, known as thermoinhibition, occurs when lettuce seeds are imbibed at temperatures above the optimum for a specific cultivar; the seeds will germinate if the temperature decreases below this level. The second phenomenon, known as thermodormancy or secondary dormancy, occurs when lettuce seeds are imbibed and maintained under high temperature for 72 hours or more, and the seeds will not germinate even if the temperature decreases to a favorable level for germination (Khan, 1980/81). In this case, a seed treatment or removal of the outer seed coverings are necessary for germination to occur (Guedes and Cantliffe, 1980; Ikuma and Thimann, 1963; Keys et al., 1975).

The embryo of lettuce seed (achene) is completely enclosed within a two-cell layer endosperm except at the radicle tip, which may be three or more cells in thickness (Borthwick and Robbins, 1928; Jones, 1974). The cell walls of lettuce endosperm are comprised largely of mannose-rich polysaccharides, probably β, 1-4 mannans (Dutta et al.,

1994; Halmer et al., 1975). These thick cells act as a barrier to seed germination, especially under high imbibition temperatures (Halmer et al., 1976). Thus, weakening the endosperm layer is a pre-requisite to radicle protrusion at high temperature (Sung et al., 1998a, 1998b).

Weakening of the endosperm has also been reported in other species, such as pepper and tomato (Dahal and Bradford, 1990; Groot and Karssen, 1992; Karssen et al., 1989; Watkins and Cantliffe, 1983). In the last few years evidence has emerged that germination of some seeds is controlled by enzymic degradation of the endosperm (Bewley, 1997a; Black, 1996). Weakening of the endosperm in the micropylar region prior to radicle protrusion was observed in anatomical studies in pepper (Watkins et al., 1985) and *Datura ferox* (Sanchez et al., 1990) and was a prerequisite to radicle protrusion for tomato seeds (Groot et al., 1988; Leviatov et al., 1995; Ni and Bradford, 1993; Nomaguchi et al., 1995). In pepper, *Datura* and tomato, endosperm weakening was suggested to be mediated by endo-β-mannanase (EC 3.2.1.78) activity in the endosperm cells (Groot et al., 1988; Sanchez et al., 1990; Watkins et al., 1985). An increase in endo-β-mannanase activity was linearly correlated with decreasing endosperm resistance to penetration (Hilhorst and Karssen, 1992). However, endo-β-mannanase activity in the endosperm cap of tomato seeds was not sufficient to permit seeds to complete germination (Toorop et al., 1996). Production of hydrolyases within the endosperm and secretion into the cell walls, causing weakening and allowing the radicle to protrude, has been proposed for many species (Bewley, 1997a; Black, 1996).

Since the lettuce endosperm cell walls are composed largely of galactomannans (Halmer et al., 1975), endo-β-mannanase might be the enzyme most likely involved in the

cell wall degradation leading to endosperm weakening and subsequent radicle protrusion. Endo-β-mannanase which hydrolyses the mannan-polymers, is produced and secreted by the lettuce endosperm (Halmer and Bewley, 1979). Endo-β-mannanase is also involved in the degradation of storage galactomannans in legumes seeds (Reid et al., 1977). In lettuce, early studies detected mannan hydrolysis only as a post-germinative event (Bewley and Halmer, 1980/81; Dulson and Bewley, 1989; Halmer, 1989; Halmer et al., 1975). Recently, Dutta et al. (1997) reported that a cell-wall-bound endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion and noted that it was regulated by the same conditions that govern seed germination.

Seed priming circumvents thermodormancy of lettuce seeds and allows germination at higher temperatures (Cantliffe et al., 1981; Guedes and Cantliffe, 1980; Khan et al., 1980/81; Valdes et al., 1985; Wurr and Fellows, 1984). The exact mechanism for seed priming to overcome thermodormancy in lettuce is not known. Guedes et al. (1981) observed a progressive loosening of the endosperm membrane during priming, possibly indicative of endosperm weakening, thus allowing lettuce seed germination to proceed at high temperature. Cantliffe et al. (1984) reported that seed priming appeared to lead to the irreversible initiation of cell elongation, thus overcoming thermodormancy. Weges et al., 1991) measured the osmotic potentials of primed lettuce seeds and reported that temperature requirement for 50 % germination was not associated with osmotic adjustment of cells. Thus, the changes that occurred in the cells were due to cell wall extensibility and was suggested as priming effect in lettuce seeds (Karssen et al., 1989).

Using a water relations analysis of the initiation of radicle growth, Bradford and Somasco (1994) suggested that the beneficial effects of priming in lettuce appeared to

occur primarily the embryo, rather than surrounding envelope tissues. In tomato, Haigh (1988) proposed that priming resulted in more rapid imbibition, increased the extensibility of radicle cell walls, and weakened the endosperm. Small et al. (1993) suggested that increased respiration and ATP production during priming might be the primary mechanism in alleviating thermoinhibition in lettuce seeds; however, seed priming did not markedly alter the pattern of respiration and ATP production (Cantliffe, 1976; Cantliffe et al., 1984). Sung (1996) observed weakening of the lettuce endosperm layer by seed priming. In another study, Sung et al. (1998b), using a puncture test, verified that priming lead to a reduction of the initial force necessary to penetrate the endosperm. Thus, the mechanism of priming in lettuce seeds that allows embryonic growth and/or causes endosperm weakening is still inconclusive.

Previous studies demonstrated that nucleic acid synthesis, protein metabolism, and ATP production are activated and/or increased in seeds during priming treatments (Bino et al., 1992; Bray et al., 1989; Coolbear and Grierson, 1979; Coolbear et al., 1990; Davison and Bray, 1991; Davison et al., 1991; Dell'Aquila and Taranto, 1986; Fujikura and Karssen, 1992; Garcia et al., 1995; Lanteri et al., 1993; Mazor et al., 1984). Activity of ATPase and acid phosphatase in peanut (Fu et al., 1988), aldolase and isocitrate lyase in pepper (Smith and Cobb, 1992), and esterase in soybean (Shatters et al., 1994) increased during seed priming. In lettuce, Khan et al. (1978) suggested that the increased activity of acid phosphatases and esterases in primed seeds resulted from activation or *de novo* synthesis. In tomato seeds, increased endo-β-mannanase activity (Karssen et al., 1989) and galactomannan-hydrolyzing enzyme (Nonogaki et al., 1992) were noted during seed priming, and the enzymes were suggested to be responsible for the endosperm weakening

leading to improved germination.

Assuming that the weakening of the endosperm must occur for lettuce seed to germinate at high temperature (Chapters 3 and 4), priming might overcome thermoinhbition and/or thermodormancy by weakening the endosperm via increased endo-β-mannanase activity. To test the hypothesis, a sensitive single-seed assay for endo-β-mannanase activity (Downie et al, 1994; Still et al., 1997) was adapted to follow enzyme activity in seeds during priming and seed germination at optimal and high germination temperatures after priming in both thermosensitive and thermotolerant lettuce genotypes.

Materials and Methods

Plant Material

Lettuce (*Lactuca sativa* L.) seeds (achenes) from thermosensitive 'Dark Green Boston' (DGB) and thermotolerant 'Everglades' (EVE) genotypes were used in this study. Thermotolerance was defined as the ability of seeds to germinate above 90% at temperatures up to 35°C in light (Guzman et al., 1992; Sung, 1996). DBG and EVE were chosen because the genetic relation between these two genotypes (Guzman et al., 1992). All seeds were produced in the same season and region of San Joaquin Valley, California in 1994. Seeds were stored at 10°C, 40% RH until used.

Seed Priming

Seeds were primed in 200 mm test tubes for three (DGB) or two (EVE) days at 15°C with constant light (~26 μmol m^{-2} s^{-1}) in an aerated solution of polyethylene glycol (PEG), at an osmotic potential of -1.2 MPa (DGB) or -1.3 MPa (EVE), (30 ml of solution g^{-1} of seed). An aquarium pump provided aeration. The air was pre-hydrated by bubbling

through water to minimize evaporation of the soaking solution. Afterward, seeds were placed in a Buchner funnel, then rinsed three times with 100 ml of distilled water and redried in an incubator at 15°C and 45% RH for two days.

Seed Moisture Content

Seed moisture content was determined by oven drying for 1 hour at 130°C, placing the seeds in a desiccator at room temperature for 20 minutes and weighing (AOSA, 1993). Samples were replicated twice.

Seed Germination

Four replications of 25 seeds were placed on two layers of 5.0 cm diameter germination paper (Anchor Paper, Hudson, WI) moistened with 3 mL of distilled water. Distilled water was added as needed to keep the filter paper moist. Blotters were covered with 5.5 cm petri dish lids and incubated at 20 and 35°C under constant light (~26 μmol m^{-2} s^{-1}) on a one-dimensional thermogradient bar (Type DB 5000, Van Dok & De Boer, B.V., Holland). Germination was defined as visible radicle protrusion through the pericarp.

Enzyme Activity

A gel-diffusion assay (Downie et al., 1994; Still et al., 1997) was used to follow endo-β-mannanase during seed priming and during germination. The methodology is described in detail in Chapter 3. After drying, lettuce seeds were placed on blotter paper moistened with distilled water and incubated for 3 hours at 4°C. This procedure was necessary in order to moisten the pericarp and endosperm before excision.

Experimental Design and Statistical Analysis

Germination tests and enzyme activity were conducted using a randomized complete block design, using in each treatment three replications. Analysis of variance (ANOVA) of data was performed by means of Statistical System (SAS) software (SAS, 1987). Treatment means were separated by the Duncan Multiple Range test.

Results and Discussion

The original seed moisture content (SMC) was 5.9 and 5.6% for DGB and EVE, respectively. Lettuce seed water uptake during seed priming followed the classical triphasic pattern (Bewley and Black, 1994) Rapid water uptake was observed in the first six hours of soaking, and the "lag phase" was markedly extended by seed priming (Figures 5-1 and 5-2). Endo-β-mannanase activity during seed priming increased between 24 and 72 hours for DGB and 24 and 48 hours for EVE of beginning of seed priming. After the end of soaking (72 and 48 hours for DGB and EVE, respectively), the SMC was 46.8% for DGB and 38.9% for EVE. No radicle protrusion was observed. Normally in water, rather than in PEG solution, SMC would be 120% after 24 hours and radicle protrusion would have occurred.

High endo-β-mannanase activity persisted in primed seeds, even following seed dry back to 5.5 and 4.9% for DGB and EVE, respectively (Figures 5-1 and 5-2). Nonogaki et al. (1992) also observed an increase of galactomannan hydrolyzing enzyme during seed priming in tomato. These authors reported that about 30% of the activity observed after the soaking period was lost during drying.

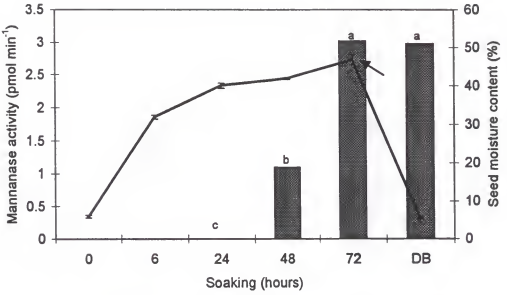

Figure 5-1. Endo-β-mannanase activity (bars) and seed moisture content (line) during priming of 'Dark Green Boston' lettuce seeds in PEG at -1.2 MPa in light at 15°C. Arrow indicates the end of soaking. DB = Dry back. Means followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$. Vertical bars indicate standard error.

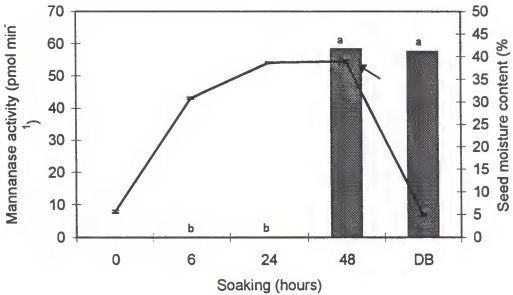

Figure 5-2. Endo-β-mannanase activity (bars) and seed moisture content (line) during priming of 'Everglades' lettuce seeds in PEG at -1.3 MPa in light at 15°C. Arrow indicates the end of soaking. DB = Dry back. Means followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$. Vertical bars indicate standard error.

Enzyme activity during seed priming was assayed from whole endosperm; enzyme localization (micropylar or lateral), however, was assayed as well during seed germination. In tomato seeds, galactomannan hydrolyzing activity was detected only in the micropylar region during seed priming (Nonogaki et al.,1992). In the present study, enzyme synthesis at the completion of lettuce seed priming reached about 65 - 70% of the total activity assayed from seeds immediately after they had visible radicle protrusion. No enzyme activity was detected in nonprimed "dry" seeds. It is also interesting to note that endo-β-mannanase activity reached 60 times higher in EVE, a thermotolerant genotype, than in DGB, a thermosensitive genotype, after 48 hours of priming. Endo-β-mannanase synthesis during priming might possibly assist in endosperm weakening, thus bypassing the need for additional wall weakening or enzyme synthesis.

At 20°C, DGB and EVE seeds germinated 100% (Table 5-1). Nonprimed seeds of EVE germinated after 10 hours and DGB after 17 hours, whereas primed seeds germinated after 3 and 5 hours, respectively. At 35°C, EVE (primed and nonprimed) and primed DGB seeds germinated 100%, while nonprimed seeds of DGB germinated 4%. Primed seeds of EVE and DGB germinated after 3 and 4 hours, respectively, whereas nonprimed seeds germinated after 10 (EVE) and 18 hours (DGB) at 35°C.

Endo-β-mannanase activity was observed during priming, and after reimbibition but before radicle protrusion when seeds of either genotype germinated under either temperature. More endo-β-mannanase activity was found in primed DGB compared to nonprimed seeds at either temperature (Figures 5-3 and 5-4). Enzyme activity in the micropylar area was high (about 50% of the total activity), considering the smaller mass of

Table 5-1. Germination of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) lettuce primed and nonprimed seeds in light at two temperatures.

Treatments	Genotype							
	DGB				EVE			
	Germination Temperature (°C)							
	20		35		20		35	
	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)	Total Germ (%)	Germ Rate (h)
Primed	100	5	100	4	100	3	100	3
Nonprimed	100	17	4	18	100	10	100	10
Significance	NS[z]	**[z]	**	**	NS	**	NS	**

[z]NS, **, Nonsignificant or significant at $P \leq 0.01$, respectively by F-test.

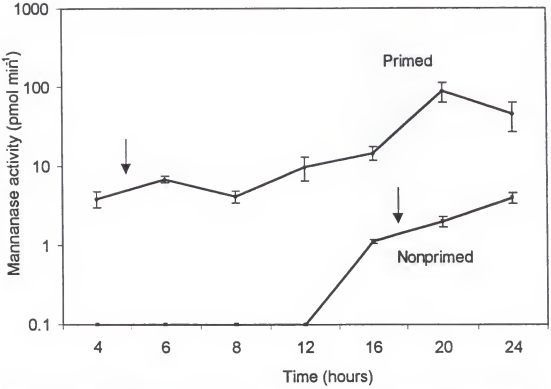

Figure 5-3. Endo-β-mannanase activity during germination of primed and nonprimed 'Dark Green Boston' lettuce seeds in light at 20°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

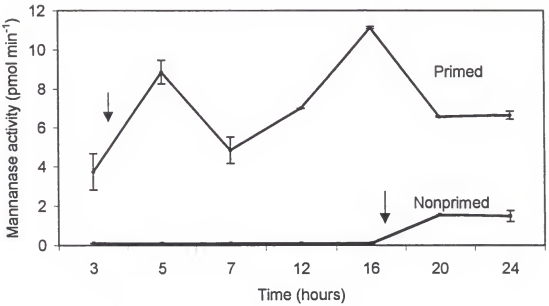

Figure 5-4. Endo-β-mannanase activity during germination of primed and nonprimed 'Dark Green Boston' lettuce seeds in light at 35°C. Arrows indicate time of radicle protrusion. Primed seeds germinated 100%, while nonprimed seeds germinated 4%. Vertical bars indicate standard error.

the micropylar tissue compared to the lateral part (Figures 5-5 and 5-6). Prior to radicle protrusion, endo-β-mannanase activity in the micropylar region was approximately three times higher in primed DGB seeds compared with nonprimed seeds under either temperature. Endo-β-mannanase activity was observed after 2 hours of imbibition in DGB primed seeds whereas in nonprimed seeds, enzyme activity was observed only after 16 and 24 hours at 20 (Figure 5-5) and 35°C (Figure 5-6), respectively.

Endo-β-mannanase was also observed in the lateral endosperm region before radicle protrusion, even at an early stage of incubation. In tomato, endo-β-mannanase is first produced in the micropylar area and after radicle protrusion, in the entire endosperm tissue (Nonogaki and Morohashi, 1996; Toorop et al., 1996; Voight and Bewley, 1996). Nonogaki and Morohashi (1996) reported some differences in the products of galactomannan hydrolysis in tomato endosperm between the pre-germinative and post-germinative enzymes, indicating that the action pattern was different between the two types of enzymes. Bewley (1997b) suggested that there are isoforms of the enzyme involved in tomato germination and other isoforms associated with post-germination cell wall mannan mobilization. This could also be true for lettuce.

After radicle protrusion, enzyme activity began to increase, possibly for endosperm carbohydrate mobilization. In general, enzyme activity was greater in seeds incubated at 20 than 35°C, corroborating results from Dutta et al. (1997). The authors observed a suppression of endo-β-mannanase activity in 'Pacific' lettuce seeds at 32°C compared with 25°C where endosperm cell walls exhibited more active autolysis. Thus, high temperature might inhibit synthesis of endo-β-mannanase.

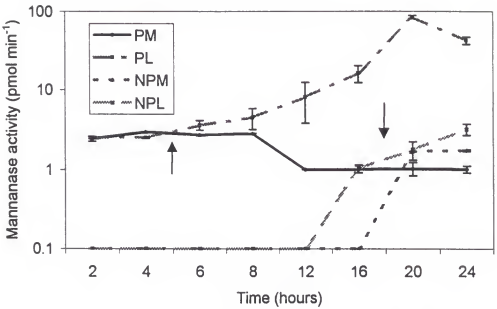

Figure 5-5. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and nonprimed (NPM = micropylar region; NPL = lateral region)'Dark Green Boston' lettuce seeds in light at 20°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

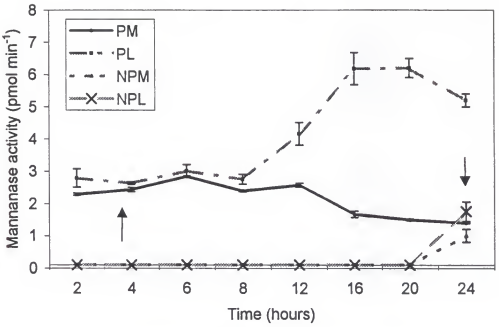

Figure 5-6. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and nonprimed (NPM = micropylar region; NPL = lateral region)'Dark Green Boston' lettuce seeds in light at 35°C. Arrows indicate time of radicle protrusion. Primed seeds germinated 100%, while nonprimed seeds germinated 4%. Vertical bars indicate standard error.

At 35°C primed thermotolerant EVE had approximately 50 fold more endo-β-mannanase during germination but before radicle protrusion than primed thermosensitive DGB. This difference is consistent with the differences observed in endo-β-mannanase activity between these genotypes during priming. In a previous study (Chapter 3) seeds of thermotolerant lettuce genotypes had greater enzyme activity before radicle protrusion at high temperature than thermosensitive genotypes. Sung et al. (1998b) verified via a puncture test that the thermotolerant genotypes had lower endosperm resistance than the thermosensitive genotypes during germination at 36°C. Moreover, thermotolerant genotypes, including EVE, were shown to have reduced physical resistance of the endosperm by weakening the cell wall and depletion of stored reserves prior to radicle protrusion (Sung, 1996; Sung et al., 1998a). Therefore, a relationship between seed germination at high temperature, a lower resistance to endosperm rupture, and an increase in endo-β-mannanase before radicle protrusion was likewise verified in the present study.

Guedes et al. (1981) observed morphological changes in lettuce seeds during priming, and reported that the membrane of endosperm cells appeared to gradually loosen. These authors concluded that this loosening might be due to endosperm weakening. Partial endosperm weakening during tomato seed priming was observed by Haigh (1988), and the mechanical resistance of the enclosing tissues also decreased during priming (Karssen et al., 1989). Haigh (1988) reported that during priming, protein body breakdown occurred in the micropylar region of endosperm cells, and those changes might be associated with endosperm weakening.

Using the same genotypes and seed lots which were used in the present study, Sung et al. (1998a) showed that primed lettuce seeds required less force to penetrate

lettuce endosperm compared with nonprimed seeds. This is consistent with the findings of the present study, e.g., higher enzyme activity in primed compared with nonprimed seeds might have to lead to this finding by Sung et al. (1998a). In response to seed priming, Sung et al. (1998a) also observed structural changes in the region micropylar (or tip) endosperm of thermosensitive cultivars at high temperature before radicle protrusion. Endosperm cells of lettuce opposing the radicle were highly vacuolated and had storage materials mobilized before radicle protrusion (Psaras et al., 1981; Sung et al., 1998a). Sung et al., (1998a) also observed that the cells at the lateral and cotyledonary end of the lettuce endosperm appeared unchanged prior to radicle protrusion at 36°C.

In EVE, although primed and nonprimed seeds had the same 100% germination, primed seeds produced about 10-fold higher levels of enzyme activity immediately before radicle protrusion than did nonprimed seeds (Figure 5-7 and 5-8). At 20°C, endo-β-mannanase activity was observed after 2 hours of imbibition in EVE primed seeds and only after 12 hours in nonprimed seeds. As previously noted, the amount of enzyme produced among individual nonprimed seed was quite variable (CV = 331%). However, germinating primed seeds exhibited a greater uniformity (CV = 25%) of enzyme activity than did germinating nonprimed seeds corroborating the results obtained in tomato (Karssen et al., 1989). The greater enzyme uniformity and activity may have contributed to uniform radicle emergence in primed seeds, regardless of temperature.

As observed at 20°C, endo-β-mannanase activity at 35°C was higher in primed compared with nonprimed seeds (Figure 5-8). High enzyme activity in the micropylar area was also observed prior to radicle protrusion in primed seeds (Figures 5-9 and 5-10).

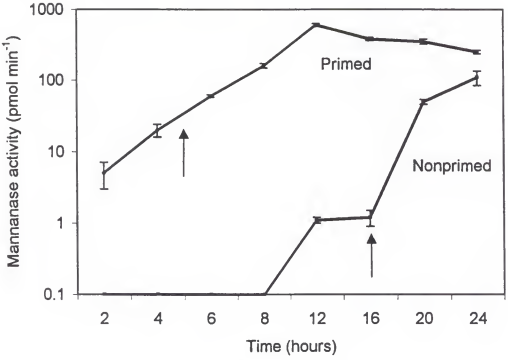

Figure 5-7. Endo-β-mannanase activity during germination of 'Everglades' primed and nonprimed lettuce seeds in light at 20°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

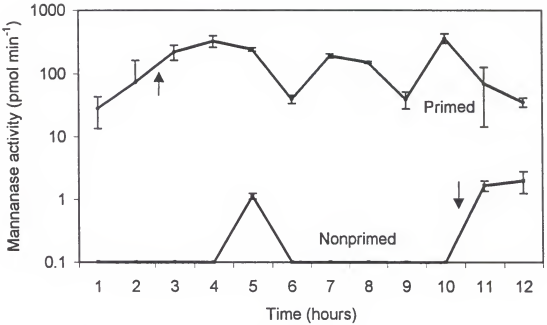

Figure 5-8. Endo-β-mannanase activity during germination of 'Everglades' primed and nonprimed lettuce seeds in light at 35°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

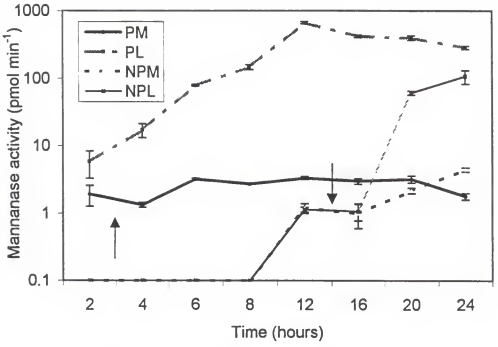

Figure 5-9. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and nonprimed (NPM = micropylar region; NPL = lateral region)'Everglades' lettuce seeds in light at 20°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

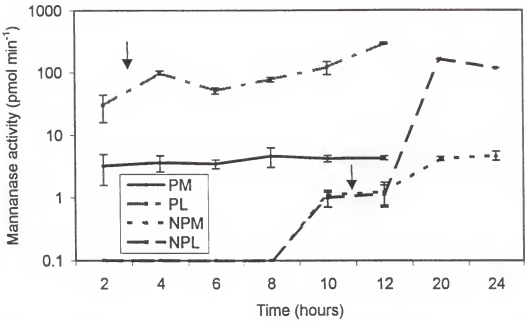

Figure 5-10. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and nonprimed (NPM = micropylar region; NPL = lateral region)'Everglades' lettuce seeds in light at 35°C. Arrows indicate time of radicle protrusion. All seeds germinated at 100%. Vertical bars indicate standard error.

Seed priming extends the lag phase of water uptake by restricting radicle emergence, and in this phase major metabolic events occur preparing the seed for phase III and radicle protrusion (Bewley and Black, 1994). Thus, a prolonged period in phase II during priming might promote endo-β-mannanase production beyond even that needed for endosperm loosening. In addition, the length of phase II is temperature dependent, however germination and endo-β-mannanase synthesis in lettuce at 15°C during the priming process proceeded at adequate rates to circumvent thermodormancy later at 35°C. Assuming that the cellular processes occurring in seeds during priming are similar to those occurring in seeds during germination (Bewley and Black, 1982), it was shown here that the synthesis of endo-β-mannanase in lettuce seeds occurred prior to the radicle protrusion. Dutta et al. (1997) reported that galactomannan-hydrolyzing activity in a cell wall extract of lettuce increased with imbibition time and was greatest just before radicle protrusion. The appearance of enzyme activity in low amounts was noted before radicle protrusion in seeds from thermotolerant lettuce genotypes in Chapter 3. A low activity of endo-β-mannanase in lettuce endosperm cell walls was suggested to be due to the limited number and specific nature of the bonds susceptible to catalysis during autolysis (Dutta et al., 1997). Thus possibly a low (basal) amount of endo-β-mannanase is adequate for lettuce endosperm weakening, thus allowing embryo growth and radicle protrusion.

In tomato an increase in endo-β-mannanase activity in the endosperm resulted from *de novo* synthesis and not to activation of pre-existing forms of the enzymes (Nonogaki et al., 1995). Synthesis of endo-β-mannanase was a prerequisite for germination in tomato. In *Datura ferox,* the micropylar endosperm region of dormant seeds contained about 15% of the endo-β-mannanase activity observed in germinating

seeds (Sanchez and de Miguel, 1997), a level presumably insufficient to cause adequate endosperm weakening (Bewley, 1997a). In the present study, levels of 1 pmol min^{-1} of endo-β-mannanase appeared adequate de Miguel, 1997), a level presumably insufficient to cause adequate for endosperm weakening, consequently leading to seed germination.

Lettuce endosperm polysaccharide composition is different in micropylar and lateral regions, where the former has a higher proportion of arabinose (Dutta et al., 1994; Chapter 3). Consequently, other enzymes (Bewley et al., 1983; Dutta et al., 1997; Halmer et al., 1975; Ikuma and Thimann, 1963; Pavlista and Valdovinos, 1975) might also be involved in endosperm weakening. The appearance of endo-β-mannanase was reported to be exclusively a post-germinative event (Bewley and Halmer, 1980/81; Dulson and Bewley, 1989; Halmer, 1989; Halmer et al., 1975). The present study shows that it is not. Perhaps the methods utilized in Bewley's studies were not adequately sensitive to detect low amounts of the enzyme. Endo-β-mannanase activity in the present and other studies was assayed using locust bean galactomannan as substrate which was not the original lettuce endosperm polymer, which could underestimate mannanase activity (Dutta et al., 1994). In the present study, using a single seed assay method, it was possible to measure enzyme levels at 1 pmol min^{-1}.

It has been suggested that lettuce seed germination was dependent on an increase in growth potential of the embryo (Nabors and Lang, 1971a, 1971b) resulting from solute accumulation (Takeba, 1980) or from an increase in cell wall extensibility leading to germination (Carpita et al., 1979). The findings of the present study support the enzymatic endosperm-weakening hypothesis as a part of the embryo growing force hypothesis for germination of lettuce at high temperature. The force of the growing embryo most likely

contributes to lettuce seed germination as does endosperm weakening. The results showed that endo-β-mannanase activity increased markedly in primed seeds before radicle protrusion, and this may be a crucial mechanism for endosperm weakening for lettuce seed germination, especially under high temperatures.

Summary

A single-seed assay for endo-β-mannanase was used to follow the activity of the enzyme during priming in lettuce seeds. The effects of seed priming on seed germination and mannanase activity at inhibitory and noninhibitory temperatures in thermosensitive 'Dark Green Boston' (DGB) and in thermotolerant 'Everglades' (EVE) lettuce genotypes were investigated. Seeds were primed at 15°C with constant light in aerated solutions of polyethylene glycol (PEG), then redried. Primed and nonprimed seeds germinated 100% at 20°C. At 35°C, EVE nonprimed and primed seeds germinated 100%, whereas nonprimed seeds of DGB germinated only 4%. During priming, endo-β-mannanase activity increased between 24 and 48 hours in EVE and between 24 and 72 hours in DGB after the beginning of osmotic imbibition. Endo-β-mannanase activity persisted in primed seeds, even following seed drying and was detected before radicle protrusion and localized partially in the micropylar region in front of the radicle tip. Higher enzyme activity was observed in primed seeds and EVE compared with nonprimed and DGB seeds. The results suggest that priming may overcome the inhibitory effect of high temperature in thermosensitive lettuce seeds due to increased endo-β-mannanase activity, possibly leading to a weakening of endosperm, thus overcoming thermodormancy.

CHAPTER 6
SEED AGING AFFECTS ETHYLENE PRODUCTION AND ENDO-β-MANANNASE ACTIVITY DURING LETTUCE SEED GERMINATION AT HIGH TEMPERATURE

Introduction

High vigor and germination are two prerequisites to achieve good stand establishment and consequently high yield and quality of the harvested crops. After seed physiological maturity, the progressive loss of seed vigor is inevitable, and the rate of vigor loss varies with genetic composition and environmental conditions. DeLouche and Baskin (1973) offered progressive and highly ordered series of events to explain the physiological basis of seed deterioration. Although the complete loss of ability to germinate is the ultimate consequence of seed deterioration, seed vigor is lost progressively. This is evident from increased sensitivity to storage conditions, a slow and unequal germination rate, a lower seedling emergence, a slow seedling growth, and an increase of frequency of abnormal seedlings (Anderson and Baker, 1983).

Physiological and biochemical changes that have been reported during seed deterioration or loss of vigor include membrane deterioration (Koostra and Harrington, 1969), solute leakage (Matthews and Bradnok, 1968), degradation of ribosomes and damage to the RNA (Roberts et al., 1973), changes in lipids (Parrich and Leopold, 1978), decline in respiration (Wooddstock et al., 1984) and ATP production (Ching, 1982), reduced ability to synthesize proteins and RNA (VanOnclelen et al., 1974), and cell destruction (Villiers, 1973). Changes in enzymes and reserve substances (Petruzelli and

Taranto, 1990), and low ethylene production (Khan, 1994) have also been correlated with loss of vigor.

Good seed vigor is necessary for tolerance to environmental stress (Heydecker, 1972), including high temperatures. For example, improved performance of preconditioned lettuce seeds at high temperature was related to high vigor (Perkins-Veazie and Cantliffe, 1984). Using two lettuce genotypes, these authors found that seed priming prevented thermodormancy in unaged but not in aged lettuce seeds. This might have been related to the reduced ability of aged seeds to produce ethylene and to develop a germination potential strong enough to remove the seed coat restraint (Khan, 1992). Early studies suggested that ethylene may enhance vigor and stimulate metabolism of some seeds. In peanut and cotton seeds, there was nearly a parallel decrease in vigor and the maximum amount of ethylene produced during germination (Ketring et al., 1974). As rapeseed vigor declined, a delay in attaining maximum ethylene production occurred. Additionally, the germination rate of aged seeds was enhanced by exogenous ethylene treatment (Takayanagi and Harrington, 1971). In lettuce, the proposed requirement for ethylene biosynthesis in seed germination might only be necessary under stressful conditions (Khan and Prusinski, 1989). It was suggested that ethylene is specifically needed for germination of thermosensitive genotypes at high temperature (Chaper 4).

Based on these findings, and the observation that ethylene production (Corbineau and Côme, 1995) and several enzymes (Walters, 1998) decline during seed aging, the inability of low vigor seeds to germinate satisfactorily at high temperature could be regulated by ethylene and/or endo-β-mannanase. The lettuce endosperm delays or prevents germination, acting as a physical barrier to radicle protrusion, especially under

high temperature. Weakening of the endosperm layer of lettuce seeds is a pre-requisite to radicle protrusion at high temperatures (Sung et al., 1998a). Dutta et al. (1997) and Nascimento et al. (1998a, 1998b, 1998c), and (Chapters 3, 4 and 5) suggested that the cell wall endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion and was regulated by the same conditions that govern seed germination. Ethylene overcomes the inhibitory effect of high temperature on lettuce seed germination (Abeles, 1986; Saini et al., 1989; Huang and Khan, 1992). Moreover, endo-β-mannanase was recently suggested be regulated by ethylene (Nascimento et al., 1998c; Chapter 4). The objective of this study was to investigate whether ethylene and endo-β-mannanase activity were affected by seed aging during germination of lettuce at high temperature.

Materials and Methods

Plant Material

Seeds of 'Everglades' (EVE) were produced in the same season and area of San Joaquin Valley, CA, in 1994. Seeds were stored at 10°C; 40% RH until used. The original seed lot was accelerated aged by placing seeds at 41°C and 100% humidity for 0, 1, 3, and 5 days, thus generating four separate seed lots varying in aging. These seed lots will be referred to as 0 (nonaged), 1, 3, and 5, indicating the duration of accelerated aging (AA) treatment.

Seed Germination

Four replications of 25 seeds were placed on two layers of 5.0 cm diameter germination paper (Anchor Paper, Hudson, WI), moistened with 3 mL of distilled water.

Blotters were covered with 5.5 cm petri dishes lids and incubated at 10, 20 or 35°C under constant light (florescent ~ 26 μmol m^{-2} s^{-1}) on a one-dimensional thermogradient bar (Type DB 5000, Van Dok & De Boer, B.V., Holland). Germination was defined as visible radicle protrusion through the pericarp. Germinated seeds were counted daily at the same time. Germination rate was calculated according to the formula Σ Ti Ni / Σ Ni, where Ni is the number of newly germinated seed at day Ti (Maguire, 1962).

Seedling Development Evaluation

Three replications of 20 newly germinated seeds (1 mm-radicle) were placed on 19 x 25 cm blotters in a horizontal line with the radicle oriented downward. Blotters were placed in a transparent plastic box at 45° from the vertical into an incubator at 20 and 35°C, under fluorescent light. After five days, length of roots and hypocotyls were measured. At this time, fresh and dry weight of roots and shoots were also determined.

Enzyme Activity

A gel-diffusion assay (Downie et al., 1994; Still et al., 1997) was used to measure endo-β-mannanase (EC 3.2.1.78) activity during seed germination. The methodology is described in detail in Chapter 3.

Ethylene Determination

Ethylene was measured after 10, 17, and 24 hours of imbibition, and the methodology is described in Chapter 3.

Experimental Design and Statistical Analysis

Germination tests, ethylene evolution and enzyme activity were conducted using a randomized complete block design, using in each treatment three replications. Analysis of variance (ANOVA) of data was performed by means of Statistical System (SAS) software (SAS, 1987). Treatment means were separated by the Duncan Multiple Range test.

Results and Discussion

At 20°C, seeds from all lots germinated 100% (Table 6-1); however, germination rate decreased with aging of 3 and 5 days compared to the control. Under stress conditions (10 and 35°C), seeds from lots aged 3 or 5 days had reduced germination at 35°C. Differences in germination rate were observed at both 10 and 35°C (Table 6-1). These results support Heydecker's conclusion that high seed vigor is responsible for tolerance to environmental stress (Heydecker, 1972).

Seed aging did not affect most of parameters of seedling development at 20°C (Table 6-2), but affected at 35°C (Table 6-3). At 35°C, germination and germination rate were reduced and correlated to a reduction in root length and hypocotyl length. Vigor in seed is defined by Association of Official Seed Analysts (AOSA) as " those seed properties which determine the potential for rapid uniform emergence and development of normal seedlings under a wide range of field conditions' (McDonald, 1980). Thus, aged seeds were considered to have reduced vigor.

Endo-β-mannanase activity was assayed in seeds that had no radicle protrusion and those that did (1 mm radicle). At 20°C, before radicle protrusion, the three-day-aged seed lot exhibited mannanase activity, whereas no activity was observed in 5-day-aged seeds

Table 6-1. 'Everglades' lettuce seed germination and germination rate from different seed aged levels in light at 10, 20, and 35°C.

Accelerated Aging	Germination (%)			Germination rate (hours)		
(days)	10°C	20°C	35°C	10°C	20°C	35°C
Nonaged	100 a[z]	100 a	100 a	24 a	24 a	24 a
1	98 a	100 a	100 a	26 a	24 a	24 a
3	98 a	100 a	73 b	46 b	43 b	26 b
5	100 a	100 a	44 c	46 b	48 c	24 a

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

Table 6-2. 'Everglades' lettuce seedling development in light at 20°C from different seed aged levels.

Accelerated Aging (days)	Root Length (cm)	Hypocotyl Length (cm)	Root Fresh Weight (mg)	Root Dry Weight (mg)	Shoot Fresh Weight (mg)	Shoot Dry Weight (mg)
Nonaged	3.26 a[z]	1.4 a	34.1 ab	1.7 a	148.6 a	9.1 a
1	2.65 a	1.4 a	30.4 b	1.5 a	135.6 a	9.3 a
3	3.21 a	1.3 a	36.7 a	2.2 a	131.6 a	9.0 a
5	3.21 a	1.3 a	35.5 ab	2.1 a	132.3 a	9.3 a

[z]Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table 6-3. 'Everglades' lettuce seedling development in light at 35°C from different seed aged levels.

Accelerated Aging (days)	Root Length (cm)	Hypocotyl Length (cm)	Root Fresh Weight (mg)	Root Dry Weight (mg)	Shoot Fresh Weight (mg)	Shoot Dry Weight (mg)
Nonaged	0.99 a	0.58 a	15 a	11 a	103 a	7 a
1	0.84 ab	0.47 a	14 a	3 b	78 a	5 b
3	0.45 c	0.44 a	9 b	10 ab	88 a	8 a
5	0.62 bc	0.47 a	8 b	5 ab	73 a	8 a

z Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

(Table 6-4). Nonaged and 1-day-aged seeds where radicle protrusion was observed had higher endo-β-mannanase activity than 3- and 5-day-aged seed lots. At 35°C, only seeds from the nonaged and 1-day-aged lots began to germinate after 12 hours of imbibition (Table 6-5) and endo-β-mannanase activity was expressed in those two treatments. Prior to radicle protrusion at 12 h nonaged seeds had higher endo-β-mannanase activity than 1-day-aged lot (Table 6-5). After 24 hours of imbibition at 35°C, differences were observed between the nonaged and the 1-day-aged lot, regardless of radicle protrusion. No endo-β-mannanase activity was detected before radicle protrusion in 3- and 5-day-aged seeds after 24 hours. Germination as recorded by radicle protrusion was generally always complete after 24 h at 35°C. Therefore, without endo-β-mannanase activity germination did not occur (Table 6-1).

A low level of endo-β-mannanase activity might affect not only the germination rate but also the percentage of germination, particularly at high temperature. Other studies have reported a high correlation between loss of viability or decrease in seed vigor and decline in activity of other enzymes (Abdul Baki and Anderson, 1972; Petruzelli and Taranto, 1990; Livesley and Bray, 1991). Loss of enzyme (e.g., amylases, proteinases, cytochome c oxidase, peroxidase, catalases, and glyceraldehyde phosphate dehydrogenase) activity has been a parameter for measuring seed deterioration, particularly enzymes associated with breakdown of food reserves (Copeland and McDonald, 1995). Biochemical vigor tests that have been used to measure loss of enzyme activity including the tetrazolium test (dehydrogenase) and the glutamic acid decarboxylase (GADA) activity test (Copeland and McDonald, 1995).

Germination rate can vary considerably among and within seed lots. Ethylene was

Table 6-4. Endo-β-mannanase activity after 24 hours from 'Everglades' lettuce seeds of different aging germinated in light at 20°C.

	Endo-β-mannanase activity (pmol min^{-1})			
	12 hours		24 hours	
Accelerated Aging	Radicle protrusion		Radicle protrusion	
(days)	No	Yes	No	Yes
Nonaged	1.7 a[z]	16.2 a	[x]	27.4 a
1	1.5 a	14.4 a	[x]	30.4 a
3	0.0 b	[y]	2.8 a	19.7 b
5	0.0 b	[y]	0.0 b	7.1 c

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

[y] No radicle protrusion at this time.

[x] All seeds germinated at this time.

Table 6-5. Endo-β-mannanase activity from 'Everglades' lettuce seeds of different aging germinated in light at 35°C.

Accelerated Aging (days)	Endo-β-mannanase activity (pmol min^{-1})			
	12 hours		24 hours	
	Radicle protrusion		Radicle protrusion	
	No	Yes	No	Yes
Nonaged	1.4 a[z]	7.2 a	2.6 a	15.2 a
1	1.1 b	2.3 b	2.5 b	14.6 ab
3	0.0 c	na[z]	0.0 c	8.2 c
5	0.0 c	na	0.0 c	4.1 d

[z] Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

measured at different time intervals, according to the time of radicle protrusion within a lot of aged seed to determine whether ethylene evolution might be associated with aging and endo-β-mannanase. Little or no ethylene was produced before radicle protrusion, and relatively high (above 20 nL h^{-1} g^{-1} seeds) amounts of ethylene were produced at the time of radicle protrusion, confirming the results obtained in previous chapters and also by Khan (1994). At 20°C, ethylene evolution was not detectable in 5-day-aged seeds after 10 and 17 hours (Figure 6-1). A small amount of ethylene was detected at 24 hours in the 5-day-aged lot. Thus, ethylene evolution decreased with increased seed aging. At 35°C, after 10 hours, 5-day- aged lot did not produce ethylene (Figure 6-2). Radicle protrusion in these seeds was at 19 hours. At 17 hours of imbibition (2 hours before radicle protrusion in 3- and 5-day-aged lots), ethylene evolution was less in the 5- than the 3-day-aged seeds.

Khan (1994) reported that ethylene production decreased in lettuce aged seeds compared to nonaged seeds. Ethylene production was highly correlated with germination and germination rate of aged seeds. However, when ACC was added during seed imbibition, ethylene evolution from aged seeds was increased regardless. As a result of this study, Khan (1994) suggested that ACC-derived ethylene production could be used as an index of vigor in lettuce seed.

In snap bean, seed deterioration was accompanied by a decline in germination and rate of ethylene production (Saminy and Taylor, 1983). Aging of seeds also decreased the ability of rapeseeds to produce ethylene (Takayanagi and Harrington, 1971). These authors suggested that further aging destroyed the ethylene producing capabilities of the seeds and apparently also destroyed the site of ethylene action, since exogenous ethylene would no longer stimulate growth. In other studies, accelerated aging caused a rapid loss

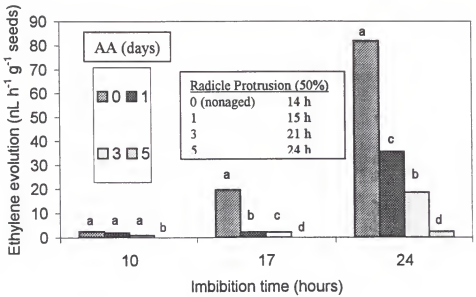

Figure 6-1. Ethylene evolution in different 'Everglades' lettuce seed lots (days of accelerated aging – AA) during germination in light at 20°C. Means in each imbibition time followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

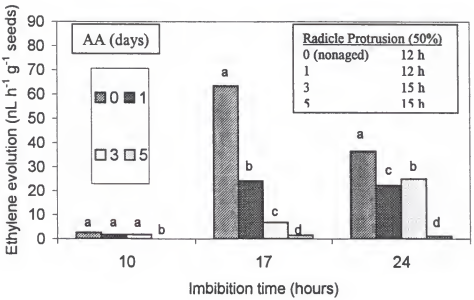

Figure 6-2. Ethylene evolution in different 'Everglades' lettuce seed lots (days of accelerated aging – AA) during germination in light at 35°C. Means in each imbibition time followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$.

of ACC oxidase activity of primed sunflower seeds (Chojnowski et al., 1997) or reduced the capacity of rice seeds to convert ACC into ethylene (Khan and Seshu, 1987).

The aging process used in the study did not affect seed viability at 20°C but did affect the ability of lettuce seeds to germinate at 35°C. Accelerated aging has been used extensively as a technique to produce seed of different vigor. (Basra, 1995). It has also been used to predict the storage potential of a seed lot (Copeland and McDonald, 1995). Accelerated aging, however, leads to increased seed moisture content during the treatment process and this may lead to increased growth of microflora. Seed moisture content is controlled under conditions of optimum storage. Thus, the results obtained using accelerated aging do not necessarily represent the real situation of natural seed deterioration. In the present study, accelerated aging was used as an approach to produce lettuce seed lots differing in vigor in order to investigate ethylene production and endo-β-mannanase activity during germination.

Low endo-β-mannanase activity prior to radicle protrusion was associated with the inhibition of lettuce seed germination at high temperature (Dutta et al., 1997; Nascimento et al., 1998a, 1998b, 1998c; Chapters 3, 4 and 5). Ethylene overcame the inhibitory effect of high temperature on lettuce seed germination (Abeles, 1986; Saini et al., 1989; Huang and Khan, 1992). An association between endo-β-mannanase activity and ethylene production during lettuce seed germination at high temperature was recently reported (Nascimento et al., 1998c; Chapters 3 and 4). The results of the present study suggest that both ethylene and endo-β-mannanase are involved in the mechanism of thermoinhibition on lettuce seeds and that accelerated aging reduces the ability of lettuce seeds to

germinate at high temperature. At 35°C, ethylene evolution and endo-β-mannanase are similarly reduced by seed aging.

Summary

The lettuce endosperm delays or prevents germination, acting as a physical barrier to radicle protrusion, especially under high temperature conditions. Weakening of the endosperm layer of lettuce seeds is a pre-requisite to radicle protrusion at high temperatures. Cell wall-associated endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion. Ethylene overcomes thermoinhibiton of lettuce seeds. The involvement of ethylene and endo-β-mannanase during germination of lettuce seeds was investigated by a gel-diffusion assay. A seed lot of lettuce 'Everglades' was aged by placing seeds at 41°C and 100% humidity for 0, 1,3, and 5 days, thus generating four seed lots varying in aging. At 20°C, seeds germinated 100%, independently of seed vigor. At 35°C, germination was lowest in seeds aged 3 or 5 days. Aged seeds also produced low ethylene levels, whereas non-aged seeds produced high ethylene levels at 35°C. More endo-β-mannanase activity was observed in nonaged seeds. The results of the present study suggest that aging can lead to reduced ethylene and endo-β-mannanase activity and thus lead to thermoinhibition in lettuce seeds.

APPENDIX

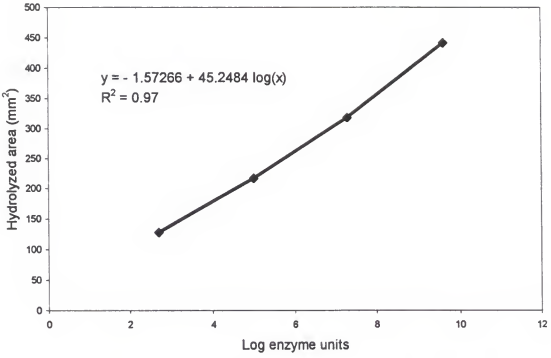

Figure 1. A representative standard curve correlating the hydrolyzed areas in the gel-diffusion assay with the logarithm of endo-β-mannanase activity.

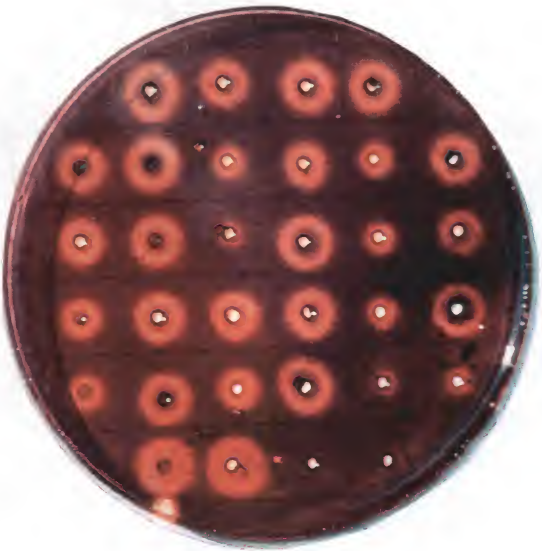

Figure 2. Assay of endo-β-mannanase in individual lettuce endosperms using gel diffusion. The four wells in the botton row represent pure enzyme (1 and 2) and buffer alone (3 and 4).

Table 1. Seed moisture content (%) after soaking (15°C, light, 3 days) and after drying (15°C, 40 % RH, 3 days) of lettuce 'Dark Green Boston' (DGB) and 'Everglades' (EVE) lettuce seeds during priming in PEG solutions, PEG+ACC (10mM), PEG+AVG (10mM), and PEG+STS (20mM).

Priming	Genotype			
	DGB		EVE	
	Seed Moisture Content (%)			
	After soaking	After drying	After soaking	After drying
PEG	42.3 a[z]	4.9 b	39.6 a	4.7 b
PEG + ACC	41.1b	5.3 b	39.3 a	6.0 a
PEG + AVG	40.3 b	4.7 b	38.7 a	6.2 a
PEG + STS	42.8 a	6.5 a	37.2 a	6.2 a

[z]Means within a column followed by the same letter were not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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